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VOL. III NOVEMBER, 1941 No. 1

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Nihil Obstat Raymond Stoll, S.T.D.
Imprimatur John T. McNicholas, Archbishop of Cincinnati
The Nihil Obstat and Imprimatur neither favor nor oppose opinions or theories here recorded. They are declarations that there is nothing in the Studies of the Institutum Divi Thomae contrary to faith or morals.

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George Speri Sperti, *Director*
Cletus A. Miller, *Dean*

THE RELATIVE OSMOTIC VALUE OF ENLARGING CELLS*

By WILLIAM A. BECK

INTRODUCTION

In the early work of these laboratories on the study of the effect of auxins on plants, Helianthus seedlings served as test objects. Later various phases of tissue and cell development were determined in delimited regions of the hypocotyl of these seedlings.[1]

In a detailed study of the hypocotyl,[2] it was shown that the bending produced during geotropic response occurs at a sharply defined point located 2.5 cm. below the base of the cotyledons. Before reaching this point it is initiated in the younger cells, and moves progressively downward from the younger cells to the older cells until this point is reached. The experiments gave evidence that the growth by cell enlargement is a physiological activity involving the simultaneous production of solute by the protoplasm and deposition of material in the cell wall in such a manner that the younger cells are more ductile than the older cells. However, the older cells do not cease to be elastic. The actual enlargement of the epidermal cells and of the cortical cells was determined.[3] The epidermal cells, taken from the region 45 – 50 mm., were ten times the size of those in the region 0 – 5 mm. from the base of the cotyledons.

From the data on hand it is evident that no proliferation of cells occurs in regions farther down than 1 or 2 mm. from the base of the cotyledons.

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*Research conducted under the Sir Charles F. Williams Grant.

[1]Beck, W. A., and Donnelly, Sr. M. W., These Studies, 2 (1939): 179.

[2]Beck, W. A., and Donnelly, Sr. M. W., These Studies, 2 (1939): 259.

[3]Beck, W. A., Schocken, K., and Donnelly, Sr. M. W., These Studies, 2 (1938): 107.

The difference in the physiological state of the cells in different phases of enlargement is, of course, of prime interest when studying the effects produced by auxins on the protoplasm. Elsewhere the author has shown that variation in the osmotic value (O_g) of the cells or tissues serves as an indicator of changes in the physiological acitivity of these cells or tissues.[4] It was therefore naturally suggested to determine the osmotic value of the cells whose enlargement was known, i.e., the epidermal cells in the zones 0 – 5 mm. and 45 – 50 mm. from the base of the cotyledons.

EXPERIMENTAL

The term "osmotic value" is chosen from the system devised by Ursprung and Blum.[5] The osmotic value of a cell or tissue is expressed as the molar concentration of the agent which produces incipient plasmolysis.[6]

For reasons previously determined[7] carefully purified sucrose was used as the plasmolysing agent. The quantity of solution employed for the immersion of the sample used for the final tests was never less than 20 cc. so that no appreciable change occurred by dilution when water was extracted from the cell sap.[7, 8]

The seedlings were raised from the author's own stock of selected seeds. In all his experiments with Helianthus the seeds used were less than one year old and of the same strain which he and his collaborators have been propagating for the last four years. German peat and quartz sand, previously

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[4]Beck, W. A., *Protoplasma*, **8** (1929): 70; **23** (1935): 203; *Plant Physiol.*, **6** (1931): 315.

[5]Ursprung, A., and Blum, G., *Biol. Zentr.*, **40** (1920): 193.

[6]Beck, W. A., *Plant Physiol.*, **3** (1928): 413. *Cf.* p. 419.

[7]Beck, W. A., *Protoplasma*, **1** (1926): 15.

[8]Wiggans, R. G., *Am. J. Botan.*, **8** (1921): 30.

washed and sterilized, was used as substratum. Before planting, the substratum was moistened with Knop's solution. Further watering was unnecessary. A temperature of 85° F. and a relative humidity of 90 per cent were maintained.

In spite of all precautions, the seedlings of the same age were rarely of exactly the same length. From the data thus far collected the author has a number of indications which point toward the causes of individual differences of seedlings, but not sufficient to give an unequivocal answer. The seedlings were 90 hours old and most of them from 65 to 70 mm. high. Smaller or larger ones were discarded. The regions from which the samples were taken were 0 – 5 and 45 – 50 mm. below the cotyledons.*

RESULTS

The results obtained are arranged and recorded in the table for ready comparison. In some cases the time consumed in testing the cells of one region was fairly long before a final answer was obtained, so that the plant was discarded without taking a sample from the other region. This was necessary because the culturing conditions in the laboratory were different from those in the culture chamber. Blanks appear in the table when both regions could not be tested on the same plants. For more than half of the plants tests were made in both regions of the same specimen. In all of these cases the O_g of the cells of the upper zone was higher than that of the cells of the lower zone. The O_g of the lower region was never as great as any value given for the upper zone. The average O_g for the upper zone was 0.224 M and for the lower zone 0.193 M. The difference in favor of the upper zone (0.031 M) is significant.

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* *Cf.* THESE STUDIES, 2 (1939): 107.

TABLE

Osmotic Value of the Epidermal Cells of Helianthus Seedlings

Plant Number	Osmotic Value at Incipient Plasmolysis in $M/C_{12}H_{22}O_{11}$ (O_g)		
	0 – 5 mm. below Cotyledons	45 – 50 mm. below Cotyledons	Difference
1	.....	0.215	.....
2	.....	0.210	.....
3	0.225	.....	.....
4	0.225	0.200	0.025
5	.....	0.200	.....
6	0.220	.....	.....
7	0.220	0.195	0.025
8	0.220	0.195	0.025
9	0.225	.....	.....
10	0.225	.....	.....
11	0.220	0.195	0.025
12	0.220	0.195	0.025
13	.....	0.195	.....
14	0.225	.....	.....
15	0.225	.....	.....
16	0.225	0.195	0.03
17	0.225	0.195	0.03
18	0.225	0.190	0.035
19	0.225	0.190	0.035
20	0.225	0.190	0.035
21	0.225	0.190	0.035
22	0.225	0.190	0.035
23	0.225	0.180	0.045
24	0.225	0.180	0.045
25	.....	0.180	.....
26	.....	0.190	.....
Average	0.224	0.193	0.031

DISCUSSION

The values obtained are in general of the order that might have been expected from results obtained on many other plants.[6] Being herbaceous the values should be relatively low. They are, in fact, lower than any values previously recorded. The only other case in which a very low value (0.25 M) was found, which however, is higher than any value recorded in the present work, was in the lower epidermis of *Euphorbia lathyrus*.[6] The fact that the seedlings were raised at a temperature of 85° F. helps to account for the relatively low O_g recorded, since, within limits, high temperature tends to decrease the O_g. The high relative humidity would not tend to make the O_g relatively low.[4]

It goes without saying that the suction tension of the upper zone must be greater than that of the lower zone, since water is certainly supplied to the upper zone from below. We may not conclude, however, from the higher O_g of the upper zone alone that the suction tension of that zone must be greater, since neither the wall pressure nor the normal turgor have been determined. It may be stated without fear of error, however, that the higher concentration of the cell sap at incipient plasmolysis in the upper region favors a higher suction tension in the upper cells.

The difference which was significant in every individual case, as well as the average difference, was not enough to maintain a sufficiently high suction tension during the ten-fold enlargement of the cells previously established. It follows that as the cells enlarged, more solute was actually produced by the protoplasm in keeping with the increment of the capacity, so that the suction tension actually became somewhat less in time. While no quantitative expression for the varying suction tension can be given until the actual turgor and wall pressure

are determined for these zones, it is interesting to note that the O_g values obtained are entirely in harmony with what might have been expected if we take into account the changing ductility and elasticity of the cell wall and the production of solute during the cell enlargement.

From the values given in the tables it becomes evident that the larger and older cells do not have a proportionately lower O_g than the younger and smaller cells, which indicates that solute must have been produced. The amount actually produced in growing epidermal cells is published elsewhere.[9]

It is well known that there is a gradient of the suction tension of the cells from the base of the hypocotyl to the top. This is true in spite of the fact that the older cells contain more solute than do the younger cells. The apparent paradox is easily explained if it is admitted that the older cells have more rigid walls and offer greater wall pressure so that, according to the law of Ursprung (which states that the wall pressure is given by the suction tension of the contents of the cell less the wall pressure) the suction tension of the older cells is quickly reduced upon the influx of water. These notions are in agreement with the findings of Ursprung and Blum,[10] who proved that the suction tension is maximum in the most actively growing region of young roots and inferred that the active growth of the wall is a part of the complex process of growth by cell enlargement. They are also in agreement with the findings of Heyn,[11] who proved that the growth-promoting substance induces increased extensibility in the cell wall. Furthermore, they are in agreement with the results obtained

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[9]Beck, W. A., *Plant Physiol.*, **16** (1941): 637.

[10]Ursprung, A., and Blum, G., *Jahrb. wiss. Botan.*, **63** (1924): 1.

[11]Heyn, A. J. N., *Protoplasma*, **19** (1933): 78.

by Söding,[12] who concludes that the wall grows by intussusception and that the first phase of growth is irreversible.

SUMMARY AND CONCLUSION

To obtain further evidence of the activity of the protoplasm during the process of cell enlargement the osmotic value of the epidermal cells of the hypocotyl of *Helianthus annuus* seedlings was determined.

The order of the recorded values (0.224 M and 0.193 M) is in harmony with previous studies of the O_g of many different kinds of epidermal cells. The O_g of the cells in the upper region was 0.031 M higher than that of the cells in the lower region.

The difference is not sufficient to account for a tenfold enlargement of the cell and thus forces the conclusions that the protoplasm must elaborate a sufficient amount of solute to make the enlargement possible without greatly reducing the concentration of the cell sap, and the cell wall must have become "fixed" to such an extent that it could not return to its earlier form in the course of plasmolysis.

The protoplasm is evidently actively engaged in the process of growth by cell enlargement as is indicated by the production of solute. This is in agreement with the findings of Strugger,[13] who showed that growth cannot be attributed to the growth of the wall alone, but involves the general activity of the protoplasm.

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[12]Söding, H., *Jahrb. wiss. Botan.*, **79** (1934): 231.

[13]Strugger, S., *Jahrb. wiss. Botan.*, **79** (1933): 406.

LITERATURE CITED

Beck, W. A., *Plant Physiol.*, **3** (1928): 413, *Cf.* p. 419, **6** (1931): 315, **16** (1941): 637; *Protoplasma*, **1** (1926): 15, **8** (1929): 70, **23** (1935): 203.

Beck, W. A., Schocken, K., and Donnelly, Sr. M. W., THESE STUDIES, **2** (1938): 107.

Beck, W. A., and Donnelly, Sr. M. W., THESE STUDIES, **2** (1939): 179, 259.

Heyn, A. J. N., *Protoplasma*, **19** (1933): 78.

Söding, H. *Jahrb. wiss. Botan.*, **79** (1934): 231.

Strugger, S., *Jahrb. wiss. Botan.*, **79** (1933): 406.

Ursprung, A., and Blum, G., *Biol. Zentr.*, **40** (1920): 193; *Jahrb. wiss. Botan.*, **63** (1924): 1.

Wiggans, R. G., *Am. J. Botan.*, **8** (1921): 30.

Original Manuscript Submitted
April 13, 1939.

THE INFLUENCE OF PROTEIN AND GLUCOSE ON THE RESPIRATORY ACTIVITY OF LIVER EXTRACTS

By SISTER MARY VERONITA RUDDY, O.P.*

Interest in the specificity of respiratory factors on the metabolic functions of cells led to a chemical investigation of liver extracts. It has been shown in our laboratories that factors which stimulate the growth, respiration and fermentation of yeast cells are apparently not identical,[1] and further that the respiratory stimulating factors obtained from different sources have a specific effect.[2] The relative respiratory effects of a number of tissue extracts have also been reported.[3] Büngeler[4] has studied the effects of several tissue extracts (especially of spleen) on the respiration of normal and tumor tissues. In our laboratories extensive investigations of the respiratory and glycolytic effects of certain tissue preparations are also in progress.

Thus far, little information has been obtained concerning the nature of the active constituents of our respiratory-active preparations beyond our apparent inability to refer them to the presence of nucleic acid-like materials[1a, 5] which appear to be the active portions of proliferation-promoting factors from

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*From the Rosary College Unit of the Institutum Divi Thomae, Rosary College, River Forest, Illinois.

[1](a) Norris, R. J., and Ruddy, Sr. M. V., These Studies, **1** (1937): 53;
(b) Norris, R. J., and Kreke, C. W., *ibid.*, **1** (1937): 137;
(c) *Cf.* also Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, **34** (1940): 1580.

[2]Ruddy, Sr. M. V., *Arch. exptl. Zellforsch.*, **22** (1939): 59.

[3]Ruddy, Sr. M. V., These Studies, **2** (1938): 21.

[4]Büngeler, W., *Frankfurt. Z. Path.*, **43** (1932): 409.

[5]Cook, E. S., and Walter, E. M., These Studies, **2** (1939): 189.

ultraviolet injured cells.[6] The present work was undertaken because qualitative tests showed the presence of carbohydrate and protein in crude liver extracts having respiratory activity. It is preliminary to a more extensive investigation into the chemical nature of the active materials. The work reported in the present paper had two objects: (1) To determine the effect of protein on the respiratory activity of the liver extracts; (2) To identify glucose as a constituent of the extracts and to note its effect on liver respiration.

EXPERIMENTAL

Preparation and Deproteinization of Extracts.

The livers of four male rats approximately 11 months old were removed immediately after the decapitation of the animal and were placed in about 5 cc. of a physiological salt solution. They were then pulped through a fine copper mesh into a weighed beaker. The pulp was mixed thoroughly, by means of a syringe, with ten times its weight of physiological salt solution.

The mixture was divided into two parts. One, called Portion I, was autoclaved for 15 minutes at 20-pound pressure. It stood at room temperature over night and was centrifuged for 15 minutes on the following morning. A second autoclaving was necessary to insure sterility. This extract was used as the undeproteinized material although some protein was removed by the heat treatments.

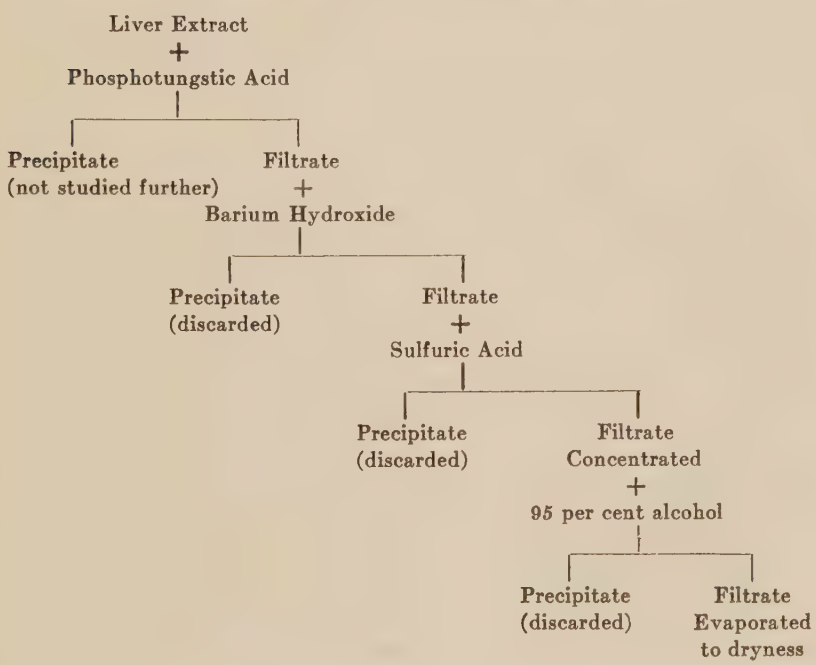
The second portion (Portion II) was placed in two sterile test tubes and stored in the ice chest. After a three-day storage period it was removed, centrifuged, autoclaved under the same

[6]Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° Congr. Intern. Chim.*, 5 (1939): 26; Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, 142 (1938): 573.

conditions as Portion I, and finally centrifuged a second time. It was deproteinized as follows:

A saturated solution of phosphotungstic acid in 5 per cent sulfuric acid was added to the centrifugate. The resultant heavy precipitate was removed by centrifuging. The supernatant fluid was treated with barium hydroxide to precipitate the excess phosphotungstic acid. Excess barium hydroxide was removed with a minimum amount of sulfuric acid. After separation of the barium sulfate precipitate by centrifugation the supernatant liquid was concentrated to half volume. An equal volume of 95 per cent ethyl alcohol was added and the resulting precipitate removed by means of the centrifuge. The liquid was taken to dryness by means of warm air. The dried residue weighed 1.018 grams. The deproteinization procedure is diagrammed in the accompanying key.

DEPROTEINIZATION PROCEDURE



The two extracts were assayed for respiratory activity on yeast according to the technique described in a previous paper.[2] The results are given in Table I. The extracts themselves did not consume oxygen. It is seen that the removal of protein increases the activity of the extracts on a weight basis. Previous experiments showed that the liver extract increased the oxygen consumption of liver tissue approximately 60 per cent.[2]

TABLE I

Respiratory Activity on Yeast by Liver Extracts before and after Deproteinization

Sample	Number of Experiments	Respiratory Period in Minutes	Concentration mg./cc.	Average Per Cent Increase in Respiration
Undeproteinized	4	20	2.5	16±5
Deproteinized	4	20	2.5	45±13

Carbohydrates.

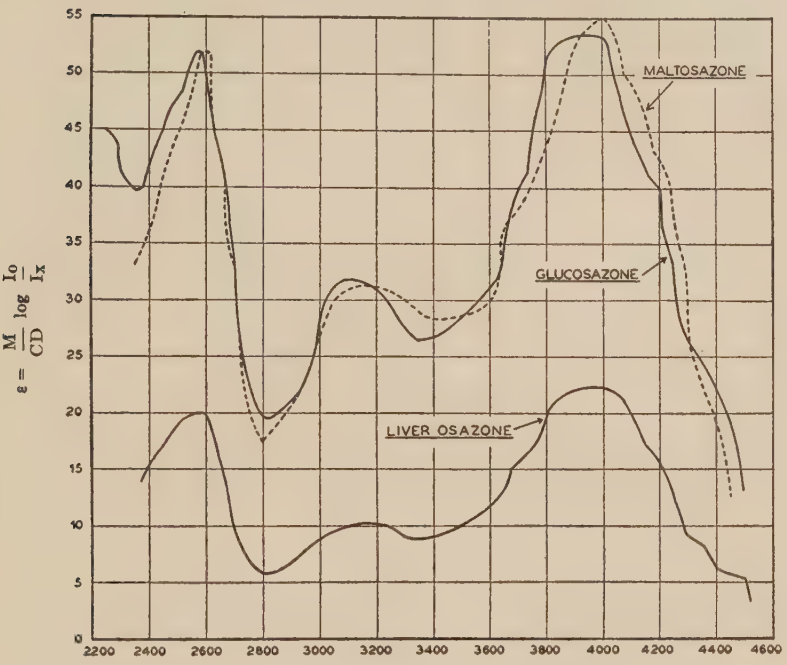
The liver extracts showed the presence of carbohydrates by the Molisch reaction. Hexoses were indicated by the Foulger guanidine reagent.[7] Pentoses also appeared to be present by the Bial test. This point will be investigated later. The hexose was isolated as the osazone by treatment of the extract with phenylhydrazine. Microscopically the osazone crystals most resembled those of glucosazone. Further identification of the osazone was made by means of ultraviolet absorption spectra.* Data on the spectra of sugar osazones may be found in Ellinger's compilation.[8] A comparison of the spectra of the liver osazone

[7]Foulger, J. H., *J. Biol. Chem.*, 99 (1932): 207.

*The author is indebted to Sister Miriam Michael Stimson for the absorption spectra.

[8]Ellinger, F., Tabulae Biologicae, Vol. 12, p. 320, Junk, The Hague, 1937.

FIG. 1—*Ultraviolet Absorption Spectra of Osazones*



and those of glucose and maltose are shown in Fig. 1. Both the liver osazone and glucosazone show a peak at about 2580 Å and another at 3950 Å although the extinction is considerably lower for the liver preparation. Maltosazone shows a similar peak, although a somewhat sharper one, at 2600 Å and a second peak at 4000 Å.

Because of the above results a quantitative estimation was made of the amount of reducing substances present. A solution of the deproteinized liver extract containing approximately 12 mg. per cc. was prepared. A total of 136.6 cc. of this solution caused only a slight reduction of Benedict's quantitative reagent. Complete reduction of this amount of the reagent

is accomplished with 50 mg. of glucose. Therefore the liver extract contained considerably less than 0.4 mg. per cc.

In view of the fact that glucose appears to be present in the liver extract, the effect of different concentrations of glucose upon the respiration of liver was determined. Kisch[9] has reported that glucose in concentrations of 0.2 per cent and M/100 does not increase the oxygen consumption of liver tissue *in vitro*. It was thought well to estimate the effects of lower and higher concentrations of glucose since concentration of substances influencing metabolism is important. Therefore the following concentrations were used: 0.02, 0.2 and 2.0 per cent. Tests were also run without the addition of glucose. The liver tissue of female rats approximately 15 months old was used. Two groups of experiments were performed, the results of which are given in Table II.

TABLE II

Effect of Glucose on Respiration of Rat Liver

	Number of Experiments	Per Cent Glucose Concentration	Average Qo_2	Average Dry Weight of Tissue in mg.
Group I	3	0.0	2.5±0.3	19.6
	4	0.02	2.4±0.4	17.9
	3	0.2	2.7±0.6	17.6
Group II	4	0.0	3.2±0.3	14.1
	4	0.2	3.6±0.5	12.5
	4	2.0	3.4±0.6	13.6

These values confirm the observations of Marsh,[10] Kisch,[9] Dickens and Greville,[11] and Quastel and Wheatley,[12] in that

[9]Kisch, B., *Biochem. Z.*, **267** (1933): 32.

[10]Marsh, M. E., *J. Nutrition*, **13** (1937): 109.

[11]Dickens, F., and Greville, G. D., *Biochem. J.*, **27** (1933): 832.

[12]Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **27** (1933): 1753.

glucose effects no change in the respiration of liver *in vitro*. The Qo_2 values are lower than those usually encountered. This may be caused by several factors: slices more than 0.3 mm. thick, oxygen uptake measured in the presence of air rather than of pure oxygen, and the sex of animal. The oxygen uptake of tissues respiring in air is less than that of tissues whose Qo_2 has been determined in an atmosphere of oxygen.[10, 13] The author is not aware of any data in the literature on the oxygen consumption of the liver of female rats but some experiments give indication that the skin of female rats has a lower Qo_2 than that of males.[14]

SUMMARY

1. Deproteinization increases the respiratory-stimulating activity of liver extracts on a weight basis. This demonstrates that protein is not responsible for the observed effects.

2. Spectroscopic and microscopic examination of an osazone prepared from liver extract indicates the presence of glucose. Its concentration is less than 0.4 mg. per cc. in the deproteinized extract.

3. Experiments confirmed the findings of others that glucose does not affect the respiration of rat liver.

BIBLIOGRAPHY

Büngeler, W., *Frankfurt. Z. Path.*, **43** (1932): 409.

Chevalier, A., and Roux, H., *Compt. rend. soc. biol.*, **118** (1935): 1348.

Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, **34** (1940): 1580.

Cook, E. S., Kreke, C.W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° Congr. Intern. Chim.*, **5** (1939): 26.

---

[13]Chevalier, A., and Roux, H., *Compt. rend. soc. biol.*, **118** (1935): 1348.

[14]Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

Cook, E. S., and Walter, E. M., THESE STUDIES, 2 (1939): 189.

Dickens, F., and Greville, G. D., *Biochem. J.*, 27 (1933): 832.

Ellinger, F., Tabulae Biologicae, Vol. 12, p. 320, Junk, The Hague, 1937.

Foulger, J. H., *J. Biol. Chem.*, 99 (1932): 207.

Kisch, B., *Biochem. Z.*, 267 (1933): 32.

Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, 142 (1938): 573.

Marsh, M. E., *J. Nutrition*, 13 (1937): 109.

Norris, R. J., and Kreke, C. W., THESE STUDIES, 1 (1937): 53.

Norris, R. J., and Ruddy, Sr. M. V., THESE STUDIES, 1 (1937): 53.

Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 27 (1933): 1753.

Ruddy, Sr. M. V., *Arch. exptl. Zellforsch.*, 22 (1939): 59.

Ruddy, Sr. M. V., THESE STUDIES, 2 (1938): 21.

Original Manuscript Submitted
July 27, 1939

RADIATIONS, CELLULAR METABOLISM AND CANCER RESISTANCE*

By GEORGE SPERI SPERTI

This paper represents a very brief review of material contained in more than fifty papers by the staff of the Institutum Divi Thomae, which either have been published or are now in press. Therefore, it is necessarily little more than a summary.

Cellular Growth and Metabolism

Basically, differences exist between malignant and normal cells. No matter what theory of the cause of cancer we accept, we must agree that these differences fundamentally involve growth and metabolism. Since growth and metabolism are characteristic of all living cells, any knowledge obtained regarding these essential properties of cells should prove of value in a study of the cancer problem when it is approached basically. This being true, for convenience in preliminary investigations, we have frequently employed cells other than those of malignant growths and cells other than those of animal origin.

Naturally, when we speak of metabolism, we think of the researches of Warburg. Many attempts have been made to confirm, and many to refute his notable work on the abnormal metabolism of malignant tissues. With its essential soundness and its inherent shortcomings, Warburg's contribution to the metabolism of tumor cells undoubtedly represents one of the greatest advances in the study of cancer in the history of

*Paper delivered by George Speri Sperti, Director of the Institutum Divi Thomae, Cincinnati, Ohio, at the Third International Cancer Congress in Atlantic City, September 12, 1939.

the problem. For reference I should like to quote Warburg's theory concerning the cause of tumors as he has stated it in his book, THE METABOLISM OF TUMOURS: "Interference with the respiration in growing cells is, from the standpoint of the physiology of metabolism, the cause of tumours. If the respiration of a growing cell is disturbed, as a rule the cell dies. If it does not die, a tumour cell results."[1] Today, most scientists do not agree with his theory of the cause of cancer. This does not mean, however, that his work is to be discarded; on the contrary, it should be supplemented.

The many controversies over Warburg's experiments on abnormal metabolism of malignant cells clearly point to the need of more fundamental research on factors influencing cellular behaviour. At the Institutum Divi Thomae we have centered our researches on these phases, and in particular on factors influencing growth, respiration, and glycolysis of cells.

Biodynes—Factors Stimulating Growth and Metabolism

It has been possible to show that three families of substances of biological origin exist which stimulate the growth, respiration, and glycolysis of living cells.[2] These substances are elaborated by cells and their production seems to be a general phenomenon characteristic of all living cells. We have been able to prove their multiple nature by showing that these substances produced by different types of cells have different characteristics. We have found that some of these substances are secretions of normal cells but that the production of some is markedly increased by injuring the cells. These substances

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[1]Warburg, O., The Metabolism of Tumours, Constable & Co., Ltd., London, 1930.

[2]Norris, R. J., and Kreke, C. W., THESE STUDIES, 1 (1937): 137; Ruddy, Sr. M. V., THESE STUDIES, 2 (1938): 21; 2 (1939): 165; *Arch. exptl. Zellforsch.*, 22 (1939): 599; Schroeder, Sr. M. P., and Cook, E. S., THESE STUDIES, 2 (1939): 247.

PLATE I

Fig. 1

113-hour tissue culture of a Segment of 12-day-old mouse embryo intestine showing increased proliferation at points (indicated by black lines) where the tissue has been cut

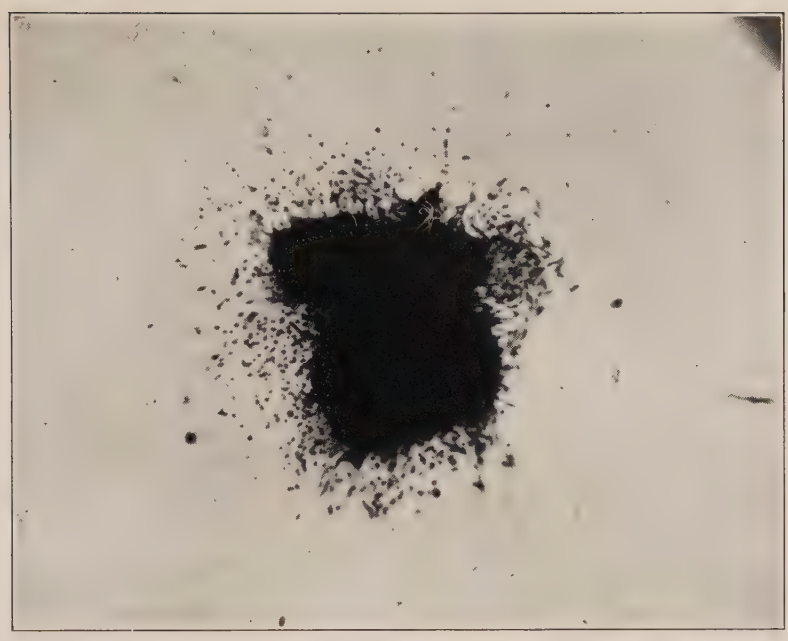

Fig. 2

41-hour tissue culture of chick embryo epithelium which has been rinsed in several changes of Drew solution

Fig. 3

41-hour tissue culture of chick embryo epithelium which has been planted without rinsing

Increased proliferation of the unrinsed tissue shows that growth factors can be washed from the tissue.

remind one of the vitamins and hormones in that they act in minute quantities. They have a direct action on many types of cells and this direct action is readily demonstrated by experiments *in vitro*. These cellular metabolic-stimulating and growth-stimulating factors appear to be of sufficiently general and fundamental physiological importance to warrant a group name. For this name we have suggested Biodynes.

Time will not permit more than very brief mention of these substances, yet, for future reference they must be discussed in this paper.

We have chosen as far as possible experiments in which we have employed radiations, in view of the particular interest of this group in this field.

Growth Biodynes.—It has long been felt that injury results in the production of factors which stimulate growth, this being one of the accepted explanations of wound repair. With this concept we thoroughly agree and have ample evidence to substantiate the contention of those who have propounded this theory.

The effect of cellular injury and destruction in promotion of growth is shown in Fig. 1, a photograph of a tissue culture of a segment of mouse embryo intestine in which increased proliferation is shown at the points where the tissue was cut.[3] Further evidence of the factors may be seen by reference to Fig. 2 and 3, showing that the growth factor can be rinsed from injured tissue. Growth factors have also been added to rinsed tissues resulting in vigorous growth.

We have been forced to recognize two types of injury: injury resulting in the *destruction* of cells; and *injury* of cells entailing no marked cellular destruction.

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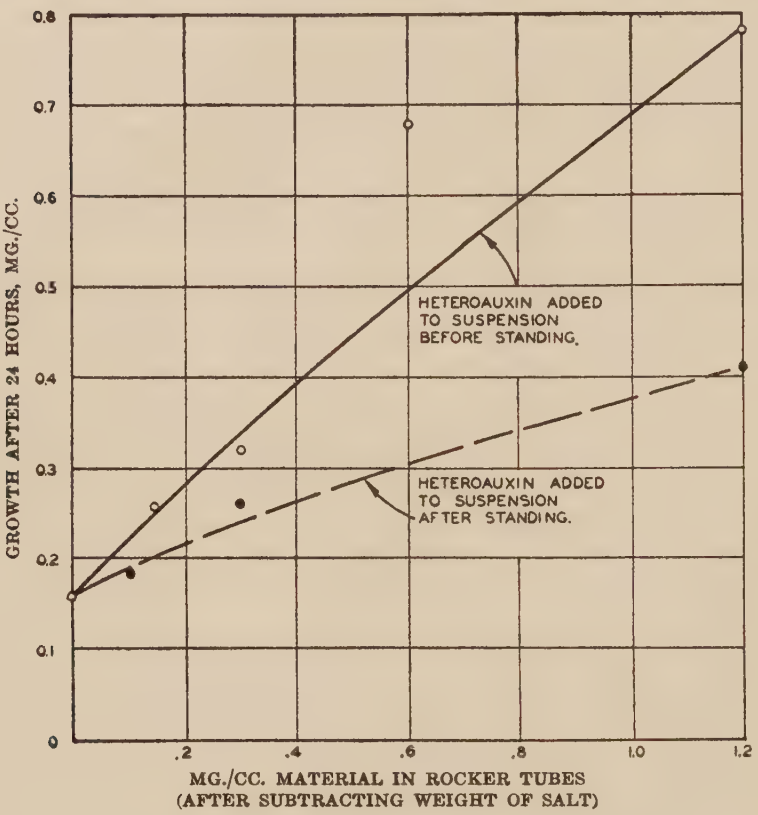
[3]Fardon, J. C., and Sullivan, W. A., THESE STUDIES, **2** (1938): 51; *Nature*, **143** (1939): 287; Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sr. M. V., *Nature*, **139** (1937): 589.

FIG. 4—*Growth-promoting Factor from Yeast Treated with Heteroauxin. Plotted on Basis of Dry Weight of Material in Fluid Added*



We recognize both a similarity and a difference between wound repair and tumor development. We recognize them both as a proliferation of cells: one, orderly and controlled; the other, disorganized and uncontrolled. The repair of a *wound*, that is, an injury which results in the *destruction* of many cells, is largely the function of the *gross organism* and is taken care of to a considerable extent by the leucocytes and cellular disintegration products. *Injury* to cells, without resulting in their death, causes the secretion of growth-

TABLE I

Crystalline 1, 2, 5, 6-Dibenzanthracene on Growth of Yeast

Molar Concentration	Per Cent Stimulation
3.6×10^{-3}	−26.4
1.8×10^{-3}	+19.6
9.0×10^{-4}	+54.0
3.6×10^{-4}	+13.2

promoting factors by the injured cells, these factors causing the multiplication of surrounding cells.[4]

We have conducted experiments with many types of cells and with many types of injuring agents including radiations, mechanical injury and chemical injury, with the result that we have been able to show that injury to a cell results in the secretion by the *injured but still living cell* of large quantities of proliferation-promoting factors.[5] Figure 4 illustrates the production of growth factors by the action of plant growth hormone.[6] The growth-stimulating effects of the carcinogenic agent 1, 2, 5, 6-dibenzanthracene can be seen by reference to Table I.[7]

[4]Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M., THESE STUDIES, **1** (1937): 163; *Nature*, **140** (1937): 643; Sperti, G. S., Loofbourow, J. R., and Lane, Sr. M. M., *Science*, **86** (1937): 611.

[5]Loofbourow, J. R., Dwyer, Sr. C. M., and Morgan, Sr. M. N., THESE STUDIES, **2** (1938): 137; Loofbourow, J. R., and Dwyer, Sr. C. M., *Science*, **88** (1938): 191; *Nature*, **143** (1939): 725; Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, **142** (1938): 573; Loofbourow, J. R., Cueto, A. A., and Lane, Sr. M. M., *Arch. exptl. Zellforsch.*, **22** (1939): 607; Loofbourow, J. R., Cook, E. S., Dwyer, Sr. C. M., and Hart, Sr. M. J., *Nature*, **144** (1939): 554.

[6]Loofbourow, J. R., and Dwyer, Sr. C. M., THESE STUDIES, **2** (1939): 155; Loofbourow, J. R., and Morgan, Sr. M. N., *J. Bact.*, **39** (1940): 437.

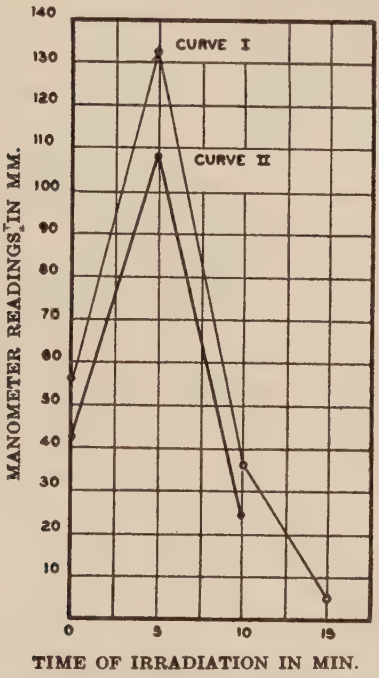
[7]Cook, E. S., Hart, Sr. M. J., and Joly, R. A., *Am. J. Cancer*, **35** (1939): 543; Cook, E. S., *Chemical Products*, London, **2** (1939): 89; Cook, E. S., and Kreke, C. W., THESE STUDIES, **2** (1939): 173, 215; Cook, E. S., and Walter, E. M., THESE STUDIES, **2** (1939): 189, 239; Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, **34** (1930): 1580.

TABLE II

Results Illustrative of Higher Yields Obtained by Prolonged, Mild Injury

Preparation	Concentration of Suspensions in mg./cc.	Irradiation Time in Hour	Concentration of Filtrate in mg./cc.	Growth Units per mg.	Growth Units per cc. Filtrate	Growth Units per g. Yeast
A	50	1.5	3.49	1.84	6.42	128
B	100	7.0	14.3	5.0	71.5	715
Filtrates from Non-irradiated Control Suspensions Corresponding to Above						
A	50	Non-irradiated less than 1% stained	1.72	0.21	0.36	7.2
B	100	Non-irradiated 1 to 2% stained	0.59	1.39	0.62	8.2

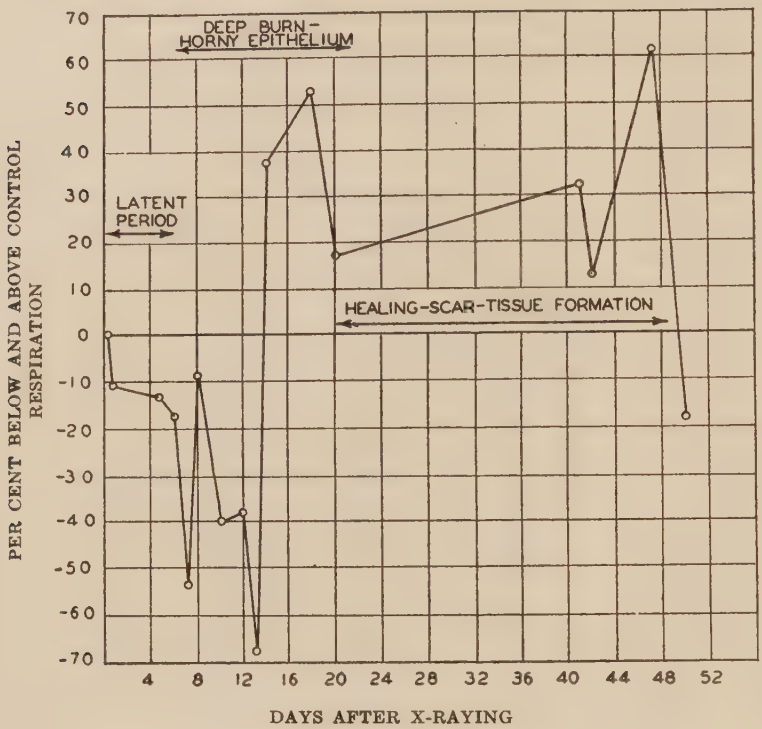
FIG. 5—*Stimulating and Depressing Effect of Ultraviolet on the Respiration of Yeast*



In Table II is cited a typical example of the difference in effect of prolonged injury and sudden death of cells on the secretions of growth factors. It will be noted that in Preparation *B* more than five times the quantity of growth factor was secreted by the cells subjected to prolonged injury than that secreted by cells which have been killed quickly, Preparation *A*.

The large secretion of proliferation-promoting factors by injured cells is in sharp contrast with the small quantities given up when the cells are killed suddenly. The fact that injury rather than the death of the cell is chiefly responsible for the secretion of proliferation-promoting factors, has been verified repeatedly in our laboratories.

FIG. 6—*Effect of X-rays on the Respiration of Mouse Skin*



The implications in the cancer problem, of course, are obvious to all who are familiar with the field.

Respiratory Biodynes.—In our researches dealing with the effect of radiations on the respiration of cells, it became apparent that two phenomena took place. Referring to Fig. 5, in which the respiration of a suspension of cells is plotted as a function of time of irradiation, it is apparent that radiations have the power both to stimulate and to depress cell respiration.[8] The explanation of the phenomena is that dead cells

[8]Fardon, J. C., Carroll, Sr. M. J., and Ruddy, Sr. M. V., THESE STUDIES, 1 (1937): 17; Fardon, J. C., and Ruddy, Sr. M. V., THESE STUDIES, 1 (1937): 41; Fardon, J. C., and Sullivan, W. A., THESE STUDIES, 2 (1938): 39.

do not respire and that cells at some point during their death processes secrete factors which stimulate the respiration of surviving cells.

The stimulation of respiration has been noted, not only through the use of ultraviolet on yeast but likewise through the use of X-rays on yeast and, of more interest to this gathering, through the effect of X-rays on normal skin.[9] (Fig. 6.)

Respiratory-stimulating biodynes have been isolated from suspensions of cells and have been shown to markedly increase the respiration of other cell suspensions to which they have been added. In some experiments we have caused cells to secrete factors, which, when added to uninjured cell suspensions, stimulated the respiration of these suspensions as much as 200 per cent.

TABLE III

Colloidal 1, 2, 5, 6-Dibenzanthracene on Growth and Respiration of Yeast

Molar Concentration		Per Cent Stimulation	
Growth	Respiration	Growth	Respiration
3.6×10^{-6}		−2.6	
2.4×10^{-6}		+17.1	
1.2×10^{-6}	3.0×10^{-4}	+25.0	+19.6
6.0×10^{-7}	1.5×10^{-4}	+24.0	−18.8
3.0×10^{-7}	7.5×10^{-5}	+33.8	−16.2
1.5×10^{-7}	3.7×10^{-5}	+8.3	−12.0

Owing to differences in growth and respiration techniques, different molar concentrations of 1, 2, 5, 6-dibenzanthracene are necessary to give the same concentration per yeast cell. These are the concentrations which are compared in the above table.

[9]Fardon, J. C., Sullivan, W. A., Brotzge, G., Loeffler, Sr. M. K., and Andrus, Sr. M. B., These Studies, 2 (1939): 203; Fardon, J. C., Brotzge, G., and Loeffler, Sr. M. K., This Issue, p. 53, Lane, Sr. M. M., and Jansen, C. H., These Studies, 2 (1939): 283.

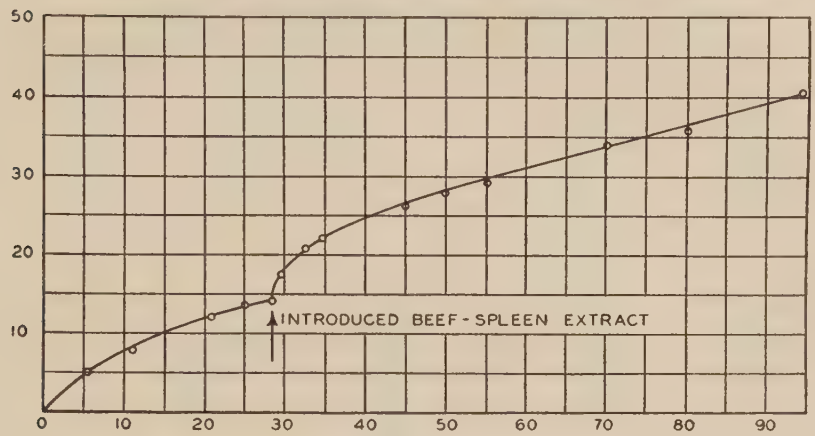
TABLE IV

The Effect of Methylcholanthrene Injection on the Respiration of Mouse Skin

Experiment Number	Age in Months	Time after Injection in Days	Control Qo_2	Experimental Qo_2	Per Cent Depression	Condition of the Experimental Skin
1	3	¾	1.10	0.34	70	Evidence of lard at site of injection
2	3	1	0.05	0.03	40	"
3	4	4	0.10	0.04	60	"
4	4	6	0.14	0.06	57	"
5	4	11	0.08	0.06	25	"
6	4	14	0.09	0.05	44	"
7	4	18	0.16	0.11	33	"
8	3	21	0.13	0.13	0	Keratic epithelium
9	4	26	0.14	0.06	57	Lard encapsulated
10	3	28	0.22	0.06	70	"
11	4	31	0.17	0.06	65	"
12	4	33	0.38	0.23	40	Evidence of hyperplasia
13	4	45	0.19	0.05	74	Normal
14	6	53	0.21	0.15	29	Tumor-like nodule near site of injection

All the mice used in these experiments were male. The methylcholanthrene was dissolved in lard.

FIG. 7—*Effect of Beef Spleen Extract on Respiration of Sarcoma*



The stimulation and depression of respiration of cells is not confined to the action of radiations but similar effects may be obtained by the use of other agents such as the carcinogenic hydrocarbons as shown in Tables III and IV.[10]

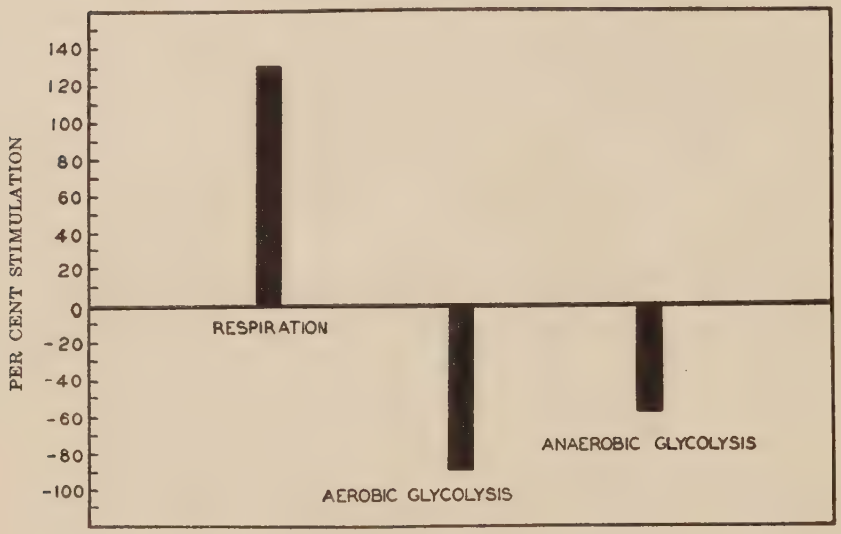
Respiratory factors are also secreted in small quantities by normal cells and undoubtedly play an important role in controlling normal tissue respiration.

The respiratory factors in some cases have been shown to be exceedingly specific, greatly stimulating the respiration of one type of normal cell and failing completely to stimulate the respiration of another type of normal cell or, again, greatly stimulating the respiration of normal tissues, and failing to stimulate the respiration of carcinoma or, again, strongly stimulating the respiration of carcinoma and stimulating only mildly the respiration of sarcoma.

Lewisohn[11] has shown that the charcoal adsorbed factor from beef spleen extract is effective in causing the regression of

[10]Cook, E. S., Hart, Sr. M. J., and Joly, R. A., *Am. J. Cancer*, 35 (1939): 543.
[11]Lewisohn, R., *Surg. Gynecol. Obstet.*, 66 (1938): 563.

FIG. 8—*Effect of Respiratory Factor from Yeast on the Respiration and Glycolysis of Methylcholanthrene Sarcoma*



Mouse Sarcoma 180. A similar extract prepared in our laboratory[12] has been shown to stimulate the respiration of Mouse Sarcoma 37S. (Fig. 7.)

Glycolytic Biodynes.—Factors which stimulate glycolysis have also been isolated from numerous types of cells. Although it is known that under some conditions glycolysis and respiration can be effected independently, a stimulation of respiration by respiratory factors can be accompanied by a simultaneous depression of glycolysis. In Fig. 8 it is shown that when the respiration of a methylcholanthrene mouse sarcoma is stimulated by the addition of a respiratory-stimulating factor, the glycolysis is simultaneously depressed.

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[12]Loofbourow, J. R., Schmieder, L., Stimson, Sr. M. M., and Dwyer, Sr. C. M., THESE STUDIES, **1** (1937): 79; Loofbourow, J. R., Norris, R. J., and Morgan, Sr. M. N., THESE STUDIES, **1** (1937): 193; Loofbourow, J. R., and Morgan, Sr. M. N., THESE STUDIES, **2** (1938): 113; Loofbourow, J. R., and Dwyer, Sr. C. M., THESE STUDIES, **2** (1938): 129.

DISCUSSION

When we recall that growth, respiration, and glycolysis are fundamental properties of all living cells and that these properties may be influenced by injury, and, when we recall that many types of injuring agents such as radiations, chemical and possibly mechanical agents, can cause cancer, the importance of these studies to the cancer problem must be obvious. Although there is a prevalent belief that in many cases cancer is related to injury, it is recognized that the majority of injuries such as cuts and burns do not, in general, result in cancer. This is exactly what we might expect in view of our experiments proving that marked changes in cellular metabolism accompany cellular *injury*, that the secretion of proliferation-promoting factors is a physiological response to injury, and that the quantity of the factor produced by a cell is much greater when the cell is subjected to prolonged injury than when the death of the cell is brought about quickly.

Two widely different agents, namely, X-rays and 1, 2, 5, 6-dibenzanthracene, have the power to depress the respiration of cells and to cause them to secrete proliferation-promoting factors. These agents, although not immediately destructive to cells, we definitely recognize as injuring agents. These, and many agents such as these, which seemingly have no immediate destructive action, are recognized as potent agents for the production of cancer.

It seems clear from our researches that carcinogenic agents have the power to injure large numbers of cells and to keep them injured over a prolonged period of time, resulting in the secretion of a large and continuous quantity of growth factor and an unbalance in metabolism. This, we feel, may be the cause of cancer.

Induced Cancer Resistance

Time will not permit more than a brief mention of the researches of Davis[13] and of Büngeler[14] on the metabolism of the normal tissues of susceptible and resistant mice. Briefly, the results are these: the average respiration of normal tissues of a strain of mice which is resistant to a transplantable tumor is considerably higher than the average respiration of the normal tissues of a susceptible strain. The importance of these findings is apparent to all when considered in the light of Warburg's discoveries concerning the unbalance in metabolism of malignant cells.

We are well aware of the fact that an animal can show a difference in degree of resistance to different types of tumors. This is a phase which is being discussed in another paper.

Resistance Induced by Injections

Murphy[15] has stated that, "Serum, tissue and organ extracts of various kinds have been injected in attempts to influence susceptibility. There have been no clear-cut results, except from the method discovered very early in the laboratory investigation of cancer: Namely, that a definite resistance to transplanted cancer may be induced by the injection of a considerable amount of living, homologous, normal cells some days in advance of the cancer inoculation. But no variation

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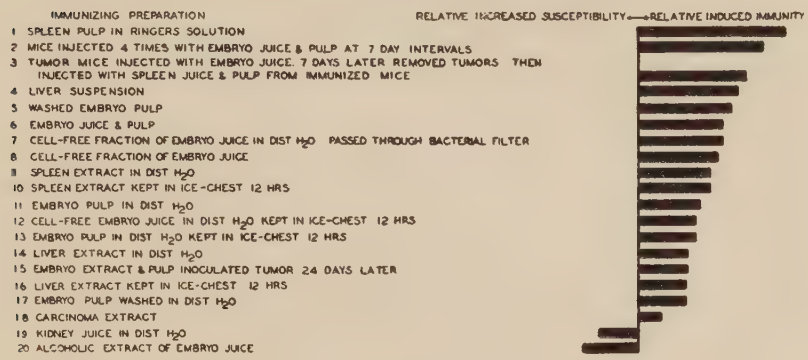
[13]Davis, J. E., *Can. Med. Assoc. J.*, **36** (1937): 27.

[14]Büngeler, W., *Frank. Z. Path.*, **39** (1930): 314; Carroll, Sr. M. J., *Arch. exptl. Zellforsch.*, **22** (1939): 592; *Nature*, **143** (1939): 684; Cook, E. S., Kreke, C. W., and Nutini, L. G., These Studies, **2** (1938): 23; Cook, E. S., and Kreke, C. W., These Studies, **2** (1938): 47; *Nature*, **142** (1938): 719; Cook, E. S., Hart, Sr. M. J., and Joly, R. A., *Science*, **87** (1938): 331; *Proc. Soc. Exptl. Biol. Med.*, **38** (1938): 169; Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° congr. intern. chim.*, **5** (1939): 26; Cook, E. S., *Chemical Products*, London, **1** (1939): 65.

[15]Murphy, J. B., Some Fundamental Aspects of the Cancer Problem, *The Science Press*, N. Y., 1937, p. 104.

FIG. 9—*Immunizing Powers of Various Tissue Preparations to the Transplantable Adenocarcinoma No. 63 of the Mouse*


IMMUNIZING PREPARATION
RELATIVE INCREASED SUSCEPTIBILITY ← → RELATIVE INDUCED IMMUNITY
1 SPLEEN PULP IN RINGERS SOLUTION
2 MICE INJECTED 4 TIMES WITH EMBRYO JUICE & PULP AT 7 DAY INTERVALS
3 TUMOR MICE INJECTED WITH EMBRYO JUICE. 7 DAYS LATER REMOVED TUMORS THEN INJECTED WITH SPLEEN JUICE & PULP FROM IMMUNIZED MICE
4 LIVER SUSPENSION
5 WASHED EMBRYO PULP
6 EMBRYO JUICE & PULP
7 CELL-FREE FRACTION OF EMBRYO JUICE IN DIST H_2O PASSED THROUGH BACTERIAL FILTER
8 CELL-FREE FRACTION OF EMBRYO JUICE
9 SPLEEN EXTRACT IN DIST H_2O
10 SPLEEN EXTRACT KEPT IN ICE-CHEST 12 HRS
11 EMBRYO PULP IN DIST H_2O
12 CELL-FREE EMBRYO JUICE IN DIST H_2O KEPT IN ICE-CHEST 12 HRS
13 EMBRYO PULP IN DIST H_2O KEPT IN ICE-CHEST 12 HRS
14 LIVER EXTRACT IN DIST H_2O
15 EMBRYO EXTRACT & PULP INOCULATED TUMOR 24 DAYS LATER
16 LIVER EXTRACT KEPT IN ICE-CHEST 12 HRS
17 EMBRYO PULP WASHED IN DIST H_2O
18 CARCINOMA EXTRACT
19 KIDNEY JUICE IN DIST H_2O
20 ALCOHOLIC EXTRACT OF EMBRYO JUICE

METHOD

The tumor tissue was cut in blocks 3 to 4 cu. mm. in volume and inoculated 7 days after the injection of the immunizing material. The tissue pulp or juice was diluted with an equal volume of Ringer solution or distilled water, and in all experiments 0.5 cc. of the material injected subcutaneously.

of the procedure has had the slightest effect on established grafts of cancer or on the development or progress of the natural disease." On the other hand several investigators including ourselves[16] were able to show conclusively that certain tissue extracts can induce resistance to transplantable tumors. (Fig. 9).

Murphy further states, "While it has been claimed that a specific anti-serum can be developed against the cancer cell, such serum has little effect on the established cancer graft and probably none on the spontaneous tumor."

In our researches published in 1933 the following experiment was described: "A number of mice bearing tumors which measured approximately 1 cm. in diameter were given an injection of embryo juice in the proximity of the tumor. Seven

[16]Fardon, J. C., *Bull. Basic Sci. Research*, 5 (1933): 5.

days later the tumors were removed surgically, small pieces of the tumor being permitted to remain in the hosts."[17] In this series of experiments 66 per cent of the control animals showed recurrences, while in the experimental series only 14 per cent showed recurrences. Experiments of this type were repeated with similar results.

Criticism may be made of the use of transplantable tumors. It is our opinion, however, that results on transplantable tumors are of prime importance since metastases merely represent multiple transplants of primary growths and hence the prevention of metastasis would reduce a malignant tumor to the status of a benign tumor.

Many researchers who have utilized spleen extracts have considered the resistance induced by these extracts to be due to a specific resistance agent found in the spleen. The fact is, however, that the extracts of many tissues have this same power. In our early experiments it occurred to us that if we were inducing a true immunity, the active agent would probably be protein in nature. The protein fraction of the cell-free extracts was tested therefore for its immunizing power with the result that the susceptibility of the animals was *increased* by this fraction. The deproteinized cell-free extract contains a factor which increases the resistance of animals to certain transplantable tumors.

By means of Warburg's respirometer technique, we have been able to show that the tissues of animals, after the injection of these deproteinized immunizing fractions, show considerably higher respiration, reminding us of the results of Davis and of Büngeler.

Other experiments have shown that when this fraction is added to tissues in respirometer flasks, respiration is markedly increased. In brief, all of this work leads one to the possible

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[17]Fardon, J. C., *Bull. Basic Sci. Research*, 5 (1933): 9.

TABLE V

Effect of Different Dosages of X-rays on the Respiration of Mouse Skin

X-rayed 2 Minutes

Experiment Number	Time after X-raying in Days	Q_{O_2}		Per Cent Stimulation
		Control	Experimental	
1	immediately	1.13	.89	− 22
3	1	.56	.82	+ 46
5	2	.87	1.06	+ 22
7	3	.47	.88	+ 86
9	4	.31	.90	+186
11	5	.47	.48	+ 2
25	6	.70	.20	− 71
13	7	.27	.25	+ 82
15	8	.34	.61	+ 79
17	9	.46	.50	+ 7
19	10	.46	.30	− 35
21	11	.76	.88	+ 16
23	12	.43	.46	+ 7

Twelve 2 to 3-month-old albino female mice were X-rayed at 45 Kv., 10 Ma. for 2 minutes. One mouse was sacrificed immediately after X-raying and one daily from each group. Each value for Q_{O_2} represents the average of two simultaneous experimental determinations.

conclusion that a relationship exists between the metabolism of normal cells and the resistance of the animal to transplantable tumors.

Resistance Induced by Radiations

Murphy and his collaborators[18] have demonstrated that X-rays have the power to induce resistance to tumor growth in

[18]Murphy, J. B., and Taylor, H. D., *J. Exptl. Med.*, 28 (1918): 1; Murphy, J. B., and Nakahara, W., *J. Exptl. Med.*, 33 (1921): 433; Murphy, J. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exptl. Med.*, 33 (1931): 299; Nakahara, W., and Murphy, J. B., *J. Exptl. Med.*, 31 (1920): 13; Norris, R. J., and Ruddy, Sr. M. V., THESE STUDIES, 1 (1937): 53; Norris, R. J., and Hart, Sr. M. J., THESE STUDIES, 1 (1937): 65.

TABLE V (Con't.)

Effect of Different Dosages of X-rays on the Respiration of Mouse Skin

X-rayed 5 Minutes

Experiment Number	Time after X-raying in Days	Qo_2		Per Cent Stimulation
		Control	Experimental	
2	immediately	.50	.51	+ 2
4	1	.61	.95	+ 57
6	2	.56	.63	+ 13
8	3	.43	1.12	+157
10	4	.79	.59	− 26
12	5	.73	.42	− 43
26	6	.44	.44	0
14	7	.67	.83	+ 25
16	8	.52	.83	+ 22
18	9	.51	.38	− 26
20	10	.50	.47	− 5
22	11	.78	.82	+ 4
24	12	.59	.61	+ 3

Twelve 2 to 3-month-old albino female mice were X-rayed at 45 Kv., 10 Ma. for 5 minutes. One mouse was sacrificed immediately after X-raying and one daily from each group. Each value for Qo_2 represents the average of two simultaneous experimental determinations.

laboratory animals as well as the power to increase their susceptibility to transplantable tumors. We are all familiar with the fact that X-rays have the power to produce cancer.

These seemingly contradictory properties of X-rays never have been adequately explained. The theories involving leucocytic infiltration have been helpful in pointing to a possible explanation of the increased resistance and the increased susceptibility induced by radiations; however, they do not seem entirely satisfactory. In an effort to shed some light on the action of X-rays, we carried on a series of experiments in which we attempted to determine if a relationship exists between cellular metabolism and the production of

TABLE V (Con't.)

Effect of Different Dosages of X-rays on the Respiration of Mouse Skin

X-rayed 25 Minutes

Experiment Number	Time after X-raying in Days	Qo_2		Per Cent Stimulation	Sex	Age in Months	Condition of Irradiated Area
		Control	Experimental				
1	⅛	.91	.91	0	♂	4	Normal
2	¾	.54	.48	−11	—	—	Normal
3	5	.47	.41	−13	♂	4	Sloughed
4	6	.84	.69	−18	—	—	Deep burn
5	7	.95	.45	−52	♂	3	———
6	8	.83	.75	−10	♀	—	———
7	10	.64	.39	−39	♂	3	———
8	12	.87	.55	−37	♂	4	Sloughed
9	13	.09	.03	−67	♂	3	Horny epithelium
10	14	.82	1.12	+37	♀	3	Sloughed-healing
11	18	.48	.74	+53	—	5	Burn
12	20	.47	.55	+17	♂	4	Partial healing
13	41	.69	.92	+32	♂	3	Cicatrized
14	42	.78	.89	+13	♂	3	Cicatrized
15	47	.55	.90	+64	♂	4	Cicatrized
16	50	1.43	1.18	−18	♂	—	Cicatrized

The mice in these experiments were X-rayed at 45 Kv., 10 Ma. for 25 minutes. Each value for Qo_2 represents the average of two simultaneous experimental determinations.

cancer by X-rays, the increase in susceptibility to cancer induced by X-rays, and the increase in resistance induced by X-rays.

It will be noted from Table V that the respiration of the normal tissues which have been treated with X-rays, in general, show an increased respiration with light dosages for the first twelve days. These experiments are cited only to show the possible relationship of respiration to cancer resistance, susceptibility, and production. Although sections of these tissues do not indicate sufficient leucocytic infiltration to explain the observed respiratory changes, the true part played by the leucocytes will have to be determined. Further researches are necessary to establish the significance of these findings.

SUMMARY

1. Tumors are known to differ from normal tissues in growth and metabolism.

2. Factors influencing cellular growth and metabolism (respiration and glycolysis) have been described. These cellular metabolic-stimulating factors appear to be of sufficiently general and fundamental physiological importance to warrant a group name—Biodynes.

3. Growth, respiratory, and glycolytic biodynes manifest a multiplicity and specificity of action.

4. The growth and metabolism of cells is definitely influenced by plant growth hormone, heteroauxin, and carcinogenic agents such as methylcholanthrene and 1, 2, 5, 6-dibenzanthracene.

5. Certain tissue extracts which, when injected, induce resistance to transplantable tumors have the power to increase the respiration of tumors and depress the glycolysis *in vitro*, thereby tending to restore the metabolism of these malignant cells to that of normal cells.

6. Facts have been presented which indicate that a possible relationship exists between the changes in metabolism of tissues brought about by X-rays and the susceptibility and resistance to tumor transplants induced by X-rays.

LITERATURE CITED

Büngeler, W., *Frank. Z. Path.*, **39** (1930): 314.

Carroll, Sr. M. J., *Arch. exptl. Zellforsch.*, **22** (1939): 592; *Nature*, **143** (1939): 684.

Cook, E. S., *Chemical Products*, London, **1** (1939): 65; **2** (1939): 89.

Cook, E. S., Hart, Sr. M. J., and Joly, R. A., *Am. J. Cancer*, **35** (1939): 543; *Proc. Soc. Exptl. Biol. Med.*, **38** (1938): 169; *Science*, **87** (1938): 331.

Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M. *Biochem. J.*, **34** (1930): 1580.

Cook, E. S., and Kreke, C. W., *Nature*, **142** (1938): 719; THESE STUDIES, **2** (1938): 47, 173, 215.

Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° congr. intern. chim.*, **5** (1939): 26.

Cook, E. S., and Walter, E. M., THESE STUDIES, **2** (1939): 189, 239.

Davis, J. E., *Can. Med. Assoc. J.*, **36** (1937): 27.

Fardon, J. C., *Bull. Basic Sci. Research*, **5** (1933): 5, 9.

Fardon, J. C., Brotzge, G., and Loeffler, Sr. M. K., THIS ISSUE, p. 53.

Fardon, J. C., Carroll, Sr. M. J., and Ruddy, Sr. M. V., THESE STUDIES, **1** (1937): 17.

Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sr. M. V., *Nature*, **139** (1937): 589.

Fardon, J. C., and Ruddy, Sr. M. V., THESE STUDIES, **1** (1937): 41.

Fardon, J. C., and Sullivan, W. A., *Nature*, **143** (1939): 287; THESE STUDIES, **2** (1938): 39, 51.

Fardon, J. C., Sullivan, W. A., Brotzge, G., Loeffler, Sr. M. K., and Andrus, Sr. M. B., THESE STUDIES, **2** (1939): 203.

Lane, Sr. M. M., and Jansen, C. H., THESE STUDIES, **2** (1939): 283.

Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

Loofbourow, J. R., Cook, E. S., Dwyer, Sr. C. M., and Hart, Sr. M. J., *Nature*, **144** (1939): 554.

Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, **142** (1938): 573.

Loofbourow, J. R., Cueto, A. A., and Lane, Sr. M. M., *Arch. exptl. Zellforsch.*, **22** (1939): 607.

Loofbourow, J. R., and Dwyer, Sr. C. M., *Science*, **88** (1938): 191; THESE STUDIES, **2** (1938): 129, 155; *Nature*, **143** (1939): 725.

Loofbourow, J. R., Dwyer, Sr. C. M., and Morgan, Sr. M. N., **2** (1938): 137.

Loofbourow, J. R., and Morgan, Sr. M. N., *J. Bact.*, **39** (1940): 437; THESE STUDIES, **2** (1938): 113.

Loofbourow, J. R., Norris, R. J., and Morgan, Sr. M. N., THESE STUDIES, **1** (1937): 193.

Loofbourow, J. R., Schmieder, L., Stimson, Sr. M. M., and Dwyer, Sr. C. M., THESE STUDIES, **1** (1937): 79.

Murphy, J. B., Some Fundamental Aspects of the Cancer Problem, *The Science Press*, N. Y., (1937): 104.

Murphy, J. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exptl. Med.*, **33** (1931): 299.

Murphy, J. B., and Taylor, H. D., *J. Exptl. Med.*, **28** (1918): 1.

Nakahara, W., and Murphy, J. B., *J. Exptl. Med.*, **31** (1920): 13.

Norris, R. J., and Hart, Sr. M. J., THESE STUDIES, **1** (1937): 65.

Norris, R. J., and Kreke, C. W., THESE STUDIES, **1** (1937): 137.

Norris, R. J., and Ruddy, Sr. M. V., THESE STUDIES, **1** (1937): 53.

Ruddy, Sr. M. V., THESE STUDIES, **2** (1938): 21, 165; *Arch. exptl. Zellforsch.*, **22** (1939): 599.

Schroeder, Sr. M. P., and Cook, E. S., THESE STUDIES, **2** (1939): 247.

Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M., THESE STUDIES, **1** (1937): 163; *Nature*, **140** (1937): 643.

Sperti, G. S., Loofbourow, J. R., and Lane, Sr. M. M., *Science*, **86** (1937): 611.

Warburg, O., The Metabolism of Tumours, Constable & Co., Ltd., London, 1930.

PREPARATION AND RESPIRATORY-STIMULATING ACTIVITIES OF SOME FRACTIONS FROM BEEF SPLEEN*

By ELTON S. COOK and ELSIE M. WALTER†

A large literature exists on the apparent relationship of the spleen to cancer development, including studies on the effects of spleen implants and spleen extracts on tumor growth, on spleen extracts as immunizing agents, and on the use of spleen extracts to cause the regression of tumors. Büngeler[1] has also published an important paper in which he demonstrated the particular potency of fairly crude spleen extracts in stimulating the respiration and depressing the glycolysis of tumors. The authors have found similar results with other extracts capable of inducing resistance.[2] Most of Büngeler's work was concerned with mouse spleens although the lesser activity of horse and ox spleen extracts was shown. Bauer *et al.*[3] have shown that an alcoholic extract of mouse spleen inhibits the development of the transplantable Ehrlich carcinoma and that the active factor can be adsorbed on animal charcoal. Recently Lewisohn[4] has reported that very concentrated preparations of beef spleen cause the disappearance of mouse sarcoma 180. Later Lewisohn[5] reported that this extract was inactive on spontaneous tumors, but regression of such tumors could be obtained with an extract prepared from the enlarged spleens of mice previously treated with the beef spleen extract.

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*Research conducted under the Sir Charles F. Williams Grant.

†Vincent Sacksteder assisted in some of the experiments.

[1]Büngeler, W., *Frankfurt. Z. Path.*, **43** (1932): 409.

[2]Schroeder, Sr. M. P., and Cook, E. S., These Studies, **2** (1939): 246.

[3]Bauer, E., Khaletzkaya, F., Lwisova, A., and Shabad, L. M., *Acta Cancrolog.*, **2** (1936): 17.

[4]Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

[5]Lewisohn, R., Third Internat. Cancer Congress, Atlantic City, Sept., 1939.

In our own laboratories considerable study has been made of the ability of various cellular extracts to stimulate the respiration and depress the glycolysis of normal and tumor tissues, and experiments are being conducted to see whether these activities may be correlated with the immunizing and tumor-regressing properties of the extracts. Our experience, as well as that of others, has shown these various properties to be widely distributed among many types of tissues. However, the predominant part which spleen has played in earlier work made it of particular interest and led to the present experiments, which must be regarded as preliminary.

EXPERIMENTAL

Spleen Fractionations.

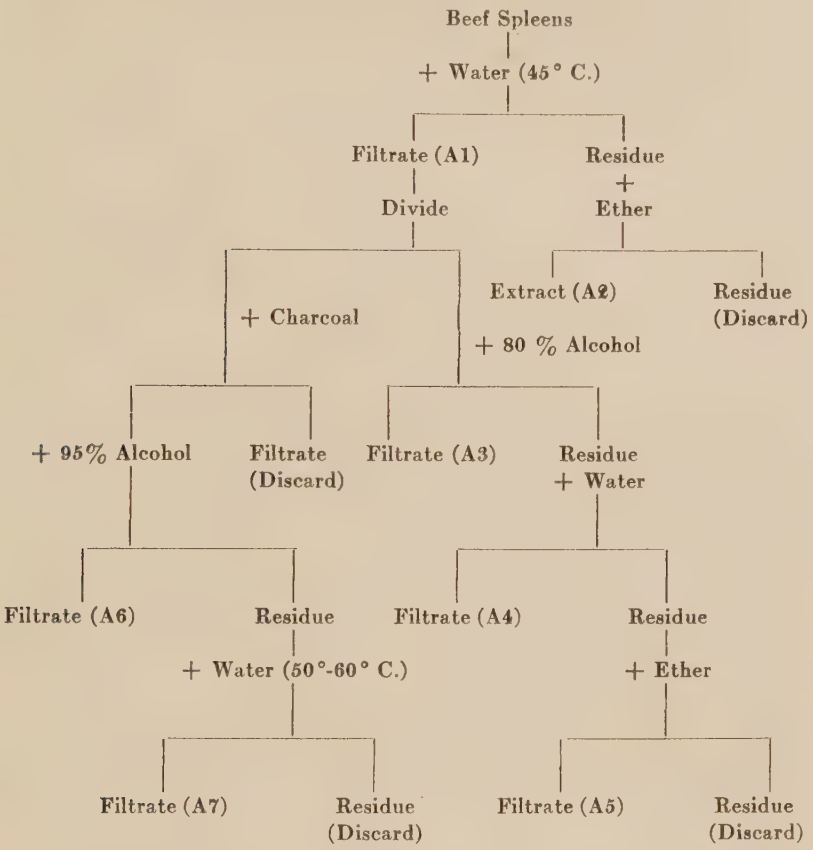
Two fractionation processes were investigated on successive batches of beef spleens. These fractionations were mainly exploratory in nature and were based partly on our experience with yeast and malt combings and partly on indications from the literature.

Method A.

Ten pounds of fresh beef spleens were skinned, cut into small pieces, and washed rapidly with cold water to remove blood. The pieces were then passed through a meat grinder and the resulting *brei* was treated with 4 liters of distilled water at 45° C. for 1 hour. The material was filtered through cheesecloth and muslin (Fraction A1). The residue was extracted with ether (Fraction A2).

The 6 liters of aqueous filtrate was divided into two equal portions. To one portion was added 12 liters of 95 per cent ethyl alcohol to give a final alcohol concentration of 80 per cent. This caused the precipitation of considerable protein. The alcoholic extract was concentrated to small volume *in*

Method A for the Fractionation of Respiratory-Stimulating Factors from Beef Spleens



vacuo at not over 40° C. The concentrated extract was finally brought to dryness in the oven at 50° C. (Fraction A3). The protein material which had been precipitated by alcohol was extracted with 1.5 liters of distilled water. This extract was also concentrated *in vacuo* and taken to dryness in the oven (Fraction A4). The insoluble material remaining after extraction with water was further extracted with ether (Fraction A5).

The second portion of the original aqueous extract was treated with 0.5 pound of activated Norit charcoal at 10° C.

TABLE I

Weights of Fractions Obtained by Method A

Fraction	Weight in Grams
A2	10.7128
A3	74.8356
A4	8.5871
A5	4.3122
A6	16.5086
A7	15.7857

(Before use the charcoal was washed with boiling distilled water, dried, and heated to red heat for 15-20 minutes.) The charcoal was filtered off with the aid of Celite and was washed three times with a total of 4 liters of 95 per cent ethyl alcohol (Fraction A6). The charcoal was then treated at 50°-60° C. with three portions of distilled water totalling 3.5 liters. The alcohol washings and water washings were separately concentrated *in vacuo* and then taken to dryness in the oven (Fractions A6 and A7).

The operations and fraction numbers are shown on the accompanying diagram. The weights of the various fractions are recorded in Table I.

*Method B**

The second fractionation was based on Method A but with certain changes suggested by experience with the earlier method. Ten pounds of fresh beef spleens were skinned, cut, washed and ground as before. The *brei* was allowed to stand over night in the icebox with 4 liters of distilled water. The mixture was then heated to 40°-50° C. for 1 hour. A sample

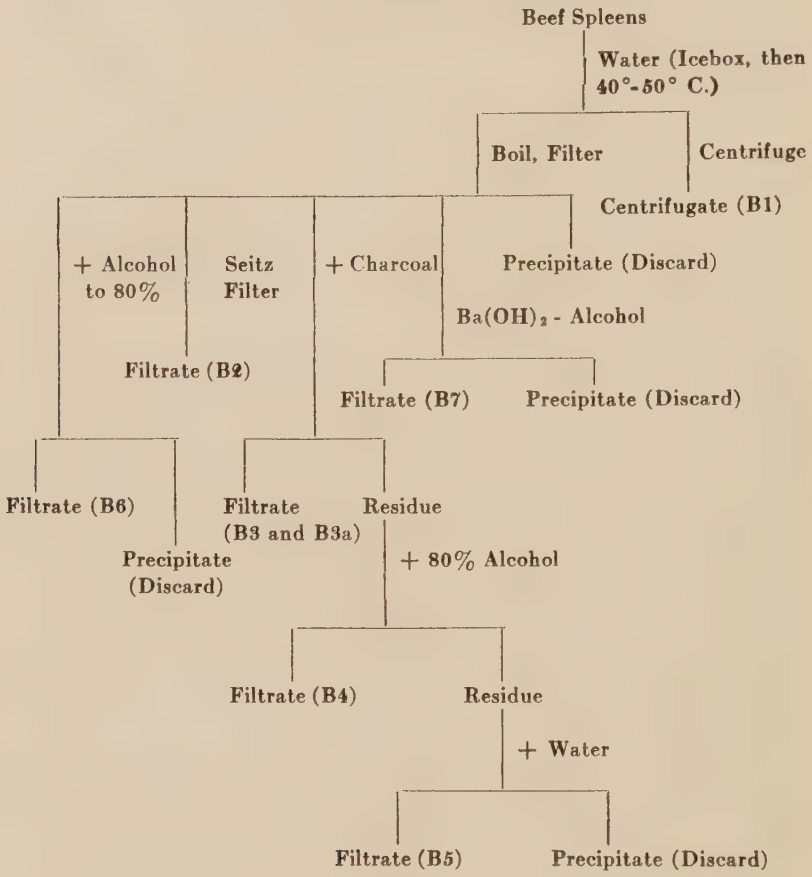
*Assistance in this fractionation was given by Sr. Cecelia Marie Dwyer, and Sr. M. Michaella Lane.

of this crude extract was centrifuged free of solid and, after adjustment of pH to 7.25 (original pH was 6.25), the supernatant liquid was passed through a sterile Seitz filter (Fraction B1).

Two liters of the filtrate were stirred with 50 g. of Norit charcoal and allowed to stand in the icebox overnight. The next morning the charcoal was filtered off but since only a little color had been removed 100 g. of Norit were added to the filtrate which was placed in the icebox for 3 hours. After filtration, 50 g. of Norit were added to the filtrate which was stirred for several minutes and again filtered. The final solution had a light straw color and a pH of 6.5. A portion of this filtrate was passed through a sterile Seitz filter after adjustment of pH to 7.4 (Fraction B3). This filtrate was concentrated *in vacuo* and taken to dryness in the oven at 50° C. Fraction B3a was prepared from this dried material by solution in distilled water, adjustment of pH to 7.3, and passage through a sterile Seitz filter. This sample differs from B3 only in that it was taken to dryness and, hence, had a known solid content. The charcoal was washed at 50°-60° C. with two 1-liter portions of 80 per cent ethyl alcohol and then with two 1.5-liter portions. The washings were rose in color, gradually lightening as they were continued. The combined washings were concentrated *in vacuo* and taken to dryness in the oven at 50° C. (Fraction B4). The charcoal was then further washed with two liters of distilled water at 50°-60° C. These washings were colorless and were taken to dryness as above (Fraction B5).

Another portion of the original filtrate after boiling (see diagram) was treated as follows: To 1.6 liters were added 8.5 liters of 95 per cent ethyl alcohol to give a final alcohol concentration of 80 per cent and the mixture was allowed to stand overnight. More protein was thereby precipitated. This was filtered off, washed with 1 liter of distilled water, and the washings were added to the alcoholic filtrate. A precipitate

Method B for the Fractionation of Respiratory-Stimulating Factors from Beef Spleens



which formed at this point was discarded. The extract was concentrated *in vacuo* and taken to dryness as usual (Fraction B6).

The remaining liter of the original filtrate after boiling was heated to 70° C. and treated with 3 liters of a solution of barium hydroxide saturated at 70° C. (315 g./liter). Then 8 liters of 95 per cent ethyl alcohol were added and the mixture was allowed to stand overnight. The precipitate was filtered

TABLE II

Weights of Fractions Obtained by Method B*

Fraction	Weight in Grams
B3a	26.6772
B4	13.2959
B5	1.1650
B6	58.8279
B7	25.6285

*Dry weights were not obtained for fractions not listed.

off and washed with 1 liter of distilled water, which was added to the filtrate. The precipitate was inadvertently discarded. The filtrate was treated with carbon dioxide to remove the barium and concentrated *in vacuo* to approximately 1 liter. This concentrate was treated with sulfuric acid until slightly acid to precipitate remaining barium. Filtration and concentration to dryness in the usual manner gave Fraction B7.

The operations and fraction numbers are shown on the accompanying diagram, and the weights of the fractions are given in Table II.

Method C.

Since the barium hydroxide precipitate from Method B was discarded, a fractionation was carried out to obtain a sample of this material. Ten pounds of beef spleens were prepared as in B and extracted overnight in the icebox with 4 liters of distilled water. The mixture was then heated to 40°-50° C. for 1 hour and finally boiled for 5 minutes and filtered. The bulk of this crude extract was treated with 80 per cent alcohol to give a deproteinized preparation (C1) which was used for experiments which are not the subject of the present paper. This sample corresponds to B6. However, 100 cc. of the original extract were heated to 70° C. and treated with 300 cc.

of a solution of barium hydroxide saturated at 70° C. Then 800 cc. of 95 per cent ethyl alcohol were added and the mixture allowed to stand overnight. The precipitate was filtered off, stirred thoroughly with approximately 800 cc. of distilled water at 50° C., and filtered. The solution was treated for 30 minutes with carbon dioxide and the barium carbonate filtered off. The filtrate was made just acid with sulfuric acid and the barium sulfate removed. The filtrate was taken to dryness at 50° C. This fraction represents the water-soluble material taken down by the barium hydroxide precipitate (C2).

The filtrate from the original barium hydroxide-alcohol precipitation was similarly treated with carbon dioxide and sulfuric acid and the remaining solution was taken to dryness at 50° C. This material constitutes the barium hydroxide filtrate (C3).

The weights of the two fractions are given in Table III.

TABLE III

Weights of Fractions Obtained by Method C

Fraction	Weight in Grams
C2	2.8664
C3	11.0589

Respiration Assays.

The various fractions were assayed for their ability to stimulate the respiration of yeast and of rat liver and skin, and some were tested on rat spleen. The methods of assay have been described previously for yeast and for rat skin and liver.[6] The experiments on spleen were carried out in the same manner as the liver determinations. Our colleagues, Fardon,

[6]Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, 2 (1938): 23.

TABLE IV

Respiratory Activity of Fractions Obtained by Method A

Fraction	Yeast Activity		Rat Tissue Activity			
	Per Cent Stimulation	Concentration mg./cc.	Per Cent Stimulation			Concentration mg./cc.
			Skin	Liver	Spleen	
A2	34.7	1.22 *	0	21	..	12.47
	23	0.61	0	0	..	6.24
A3	90	0.975	11	65	16	9.75
	63	0.488	0	57	21	4.88
A4	137	1.0	42	42	28	9.08
	52	0.5	42	21	28	4.54
A5	28	1.0	12	0	14	12.55
	21	0.5	0	0	0	6.28
A6	...	...	51	82	34	12.11
	...	...	23	56	42	6.06
	204	1.19	17	110	17	11.88
	117	0.59	23	78	0	5.95
A7	...	...	31	90	0	11.15
	...	...	19	69	0	5.58
	41	1.1	..	122	..	10.97
	32	1.23	..	132	..	12.26
A3†	130	0.97	22	81.6	..	9.75
A4†	70	1.0	13	...	..	9.68
			..	108	..	15.48
A6†	184	1.19	22	169	..	11.88
A7†	84	1.10	44	111	..	10.97

*Bracketed pairs indicate experiments on tissues of the same animal or on the same yeast suspension.

†Samples run on same yeast suspension and on tissues from same rat.

Brotzge and Loeffler, have also studied the activity on tumors of certain of the fractions prepared by Method A; this work is described in another paper in this journal.[7]

For assay, the dried fractions were made up to a concentration of 30-40 mg./cc. in sterile Ringer-phosphate-glucose

[7]Fardon, J. C., Brotzge, G. C., and Loeffler, Sr. M. K., This Issue, p. 69.

TABLE V

Respiratory Activity of Fractions Obtained by Method B

Fraction	Yeast Activity		Rat Liver Activity	
	Per Cent Stimulation	Concentration mg./cc.	Per Cent Stimulation Liver	Concentration mg./cc.
B1†	65	0.1cc. *	68	1.0cc.
	51	0.05cc.	49	0.5cc.
B2†	51	0.1 cc.	44	1.0cc.
	35	0.05cc.	33	0.5cc.
B3†	12	0.1 cc.	65	1.0cc.
	11	0.05cc.	55	0.5cc.
B3a	..	.....	85	12.7
	..	.....	100	6.3
	57	1.2	66	12.7
	39	0.6	63	6.33
B4	..	.....	72	12.3
	..	.....	74	6.2
	43	1.2	62	12.3
	30	0.6	29	6.1
B5	33	0.75	39	7.55
	22	0.38	43	3.8
B6	49	1.3	69	12.6
	35	0.63	54	6.3
B7	46	1.3	18	12.8
	47	0.64	38	6.4

*Bracketed pairs indicate experiments on tissues of the same animal or on the same yeast suspension.

†Not taken to dryness.

(0.02 per cent glucose for yeast determinations, 0.2 per cent for tissues) and any insoluble material was filtered off in weighed Gooch crucibles. The pH of the solution was adjusted to 7.3 (glass electrode) with sodium hydroxide and the solution was then passed through a Seitz bacterial filter. Preliminary experiments showed that Seitz filtration or autoclaving (20 pounds for 20 minutes) did not affect the activity.

TABLE VI

Respiratory Activity of Fractions Obtained by Method C

Fraction	Yeast Activity		Rat Tissue Activity		
	Per Cent Stimulation	Concentration mg./cc.	Per Cent Stimulation		Concentration mg./cc.
			Skin	Liver	
C1	..	....	..	57	7.37 *
	..	....	..	50	14.74 *
C2	47	1.20 *	22	24	4.90 *
	35	0.60 *	..	37	9.81 *
			26	56	12.0 *
			13	40	6.0 *
C3	145	1.41 *	11	30	14.1 *
	86	0.70 *	8	39	7.0 *

*Bracketed pairs indicate experiments on tissues of the same animal or on the same yeast suspension.

All samples were tested in manometers in the absence of tissue or yeast. In no case did the samples absorb oxygen.

The results of these assays are given in Tables IV, V, and VI. Bracketed pairs indicate experiments on tissues of the same animal or on the same yeast suspension.

DISCUSSION

The results of these assays leave much to be desired since their quantitative reproducibility is sometimes poor. This is at least partially due to the well known variations in tissues from animal to animal. In an attempt to overcome this difficulty one set of assays (those marked with a dagger in Table IV) was run with several fractions on tissues from the same rat and on the same yeast suspension. Nearly all other assays were run on tissues from different animals and on different yeast suspensions. It must also be recalled that the conditions of fractionation were almost inevitably not the

ideal ones. The procedures were for the most part exploratory and were carried out with the object of detecting in a general way the behavior of the active materials. It is obvious that results will become clear-cut only when the best conditions are determined for performing a given operation, such as adsorption. Therefore, too much significance cannot be given to the absolute quantitative activities recorded and it is fair only to compare the activities of the fractions in a very general way. The results will, however, furnish the basis for future experimental procedures. Bearing these limitations in mind, the following facts appear to emerge from the data:

1. The ether-soluble fractions (A2 and A3) have only slight stimulatory activity on yeast and little or no activity on skin and liver. This is in agreement with much of our previous experience which indicates the respiration stimulating factors to be water-soluble with the exception of certain fatty acids.[8]

2. Materials active on both yeast and tissue respiration, are not precipitated by 80 per cent ethyl alcohol (A3 and B6) but may be adsorbed to the protein so precipitated and they can be washed from the precipitate (A4). The active factors, therefore, do not appear to be protein in nature. In actual fact most of these filtrates from alcohol treatment are not quite free of protein as shown by slightly positive biuret reactions. However, the active charcoal treated fractions were protein-free by the biuret test.

3. According to previous indications from the literature, spleen fractions active in establishing tumor immunity are adsorbable by charcoal. We had previously found, in preparations from yeast, that the factor active in increasing yeast respiration is particularly well adsorbed by charcoal but that the skin-active factor can also be adsorbed, particularly by

[8]Cook, E. S., and Kreke, C. W., THESE STUDIES, 2 (1938): 47; 2 (1939): 215; *Nature*, 142 (1938): 719; Cook, E. S., and Morgan, Sr. M. N., *Biochem. J.*, 34 (1940): 15.

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more active charcoals.[9] In the present work we find materials active on yeast, skin and liver (A6, A7, B4, and B5) to be adsorbed by charcoal. It would appear that the yeast factor is particularly well adsorbed and can be eluted well with 95 per cent ethyl alcohol (A6). A second elution with water removes further yeast-active material which is less potent than the alcohol eluate, perhaps due to the presence of contaminating substances. However, this water elute (A7) possesses tissue activity approximately equal to that of the alcohol elute as far as the data allow comparison.

Under the conditions of Method B considerable active material is not adsorbed (B3a). Material, active on both yeast and liver, can be eluted from the charcoal by 80 per cent ethyl alcohol (B4) and by water (B5).

Under the conditions of the experiments charcoal treatment has not been as effective in concentrating and separating the active materials as have been similar methods with yeast extracts.[9] The treatment has, however, provided protein-free preparations, as pointed out above.

4. Both the barium hydroxide-ethyl alcohol precipitate (C2) and filtrate (C3, B7) are active on yeast, skin and liver. However, the filtrate is more active on yeast than the precipitate, while the precipitate appears to be more active on skin than the filtrate; slightly higher liver activity is also found in the precipitate. These results accord with the assays on similar fractions from yeast.[6]

SUMMARY

1. Extracts of beef spleen increase the respiration of yeast, rat liver and rat skin.

2. The active materials are water-soluble.

3. The active materials appear to be non-protein in nature.

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[9]Cook, E. S., and Walter, E. M., These Studies, 2 (1939): 189; This Issue, p. 139.

4. The active materials are adsorbed by Norit charcoal, but the conditions of adsorption must be improved to effect satisfactory concentration.

5. The yeast-active and tissue-active factors are probably distinct as suggested by apparent differences in elution from charcoal and by differences in precipitability by barium hydroxide-ethyl alcohol. These results accord with our experience with factors from other sources.

BIBLIOGRAPHY

Bauer, E., Khaletzkaya, F., Lwisova, A., and Shabad, L. M., *Acta Cancrolog.*, **2** (1936): 17.

Büngeler, W., *Frankfurt. Z. Path.*, **43** (1932): 409.

Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

Cook, E. S., and Kreke, C. W., THESE STUDIES, **2** (1938): 47; **2** (1939): 215; *Nature*, **142** (1938): 719.

Cook, E. S., and Morgan, Sr. M. N., *Biochem. J.*, **34** (1940): 15.

Cook, E. S., and Walter, E. M., THESE STUDIES, **2** (1939): 189; THIS ISSUE, p. 139.

Fardon, J. C., Brotzge, G. C., and Loeffler, Sr. M. K., THIS ISSUE, p. 69.

Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

Lewisohn, R., Third Internat. Cancer Congress, Atlantic City, Sept., 1939.

Schroeder, Sr. M. P., and Cook, E. S., THESE STUDIES, **2** (1939): 246.

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December 20, 1939.

DEPRESSED RESPIRATION PRECEDING TUMOR FORMATION*

By JOHN C. FARDON, REV. GUSTAVE C. BROTZGE, and SISTER M. KENNETH LOEFFLER, O.P.

In general it is now held that the primary difference between normal and malignant tissue is one of metabolism. It was not until the work of Warburg[1] that experimental evidence brought about a more lucid concept of this difference in metabolism. From his experiments Warburg concluded the following: "Interference with the respiration in growing cells is, from the standpoint of the physiology of metabolism, the cause of tumours. If the respiration of a growing cell is disturbed, as a rule the cell dies. If it does not die, a tumour cell results." Although the present evidence would not permit Warburg's definite conclusion that a decrease in respiration alone is the cause of cancer, there does seem to be sufficient evidence in the literature to indicate that a decrease in respiration is associated with the cause of cancer.

In accord with the opinion expressed by Warburg on oxygen deficiency are the experiments of Davis,[2] who recently showed that the natural resistance or susceptibility of animals to cancer is related to the respiration of the normal tissues of these animals. We have shown in these laboratories[3] that, after the injection of immunizing agents, the respiration is markedly increased. What seems, therefore, to hold for natural immunity seems also to hold for induced immunity.

If the hypothesis advanced by Warburg is correct, then one should be able to detect this oxygen deficiency in tissue

*Research conducted under the Alex Meis Grant.

[1]Warburg, O., *J. Cancer Research*, 9 (1925): 148.

[2]Davis, J. E., *Can. Med. Assoc. J.*, 36 (1937): 27.

[3]Fardon, J. C., Brotzge, G., and Loeffler, Sr. M. K., This Issue, p. 61.

which has been subjected to conditions favoring tumor formation, in other words, to a carcinogenic agent.

Dyadyuslia,[4] treating mice with coal tar, observed that the anaerobic glycolysis of the skin was considerably increased in the first month of cancerization, but that the respiration was found lowered. In mice treated with 1, 2, 5, 6-dibenzanthracene he found the respiration normal for the first two months. Pourbaix,[5] working with brain, spleen, and liver, found that low concentrations of 1, 2, 5, 6-dibenzanthracene and 3, 4-benzpyrene depressed the respiration of these tissues *in vitro*. The work of Cook[6] in these laboratories may also be of interest. Using 1, 2, 5, 6-dibenzanthracene, Cook found that growth and respiration of yeast could be stimulated or depressed, depending upon the concentration of the hydrocarbon. Fardon and Sullivan[7] in these laboratories, studying the effect of X-ray burns on the respiration of mouse skin, found an initial period of depression, which was later followed by a period of stimulation. Though observations were not continued over an extended interval, no tumors resulted from this X-ray treatment.

That depressed respiration alone cannot be considered as the primary factor in tumor induction may be inferred from other experiments. Cook,[8] for example, in a discussion on respiratory stimulators and depressors, points out that preservatives (sodium benzoate), antiseptics, and certain cosmetic lotions and creams depress the respiration of skin. Deficiency in vitamins B_1, G, D, and A also lowers tissue respiration.

It must be conceded that in reviewing such experimental results it is unlikely that all agents which are capable of

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[4]Dyadyuslia, G. F., *J. Med., Ukraine*, **7** (1937): 131.

[5]Pourbaix, Y., *Compt. rend. soc. biol.*, **110** (1932): 1015; **112** (1933): 1222; **113** (1933): 930; **115** (1934): 1738.

[6]Cook, E. S., Hart, Sr. M. J., and Joly, R. A., *Am. J. Cancer*, **35** (1939): 543.

[7]Fardon, J. C., and Sullivan, W. A., *Nature*, **143** (1939): 287.

[8]Cook, E. S., *Chemical Products*, **1** (1939): 65; **2** (1939): 89.

lowering the respiration of tissue finally result in the production of tumors. However, it must be admitted that there seems to be a certain relation between tumor induction and depressed respiration.

The purpose of this paper is to determine if a lowering of respiration as a result of the injection of a carcinogenic hydrocarbon takes place *prior* to tumor development.

EXPERIMENTAL

A total of 14 male albino stock mice, ranging in age from 3 to 4 months, were used in these experiments. The mice received a single subcutaneous right lateral injection of 0.5 mg. of methylcholanthrene in 0.25 cc. of lard. The animals were then placed in single-compartment cages and given the usual diet. To aid the diffusion and absorption of the lump of lard, Andervont's[9] method of applying pressure to the mass formed at the site of injection was employed from time to time. The respiration experiments were begun 18 hours after injection. The animal was prepared by shaving the area of skin to be used and was sacrificed by decapitation. The right lateral and slightly ventral portion of the skin was used as experimental, discarding a small section immediately surrounding the injection site. The left lateral and slightly ventral portion was used as control. A narrow strip of skin, 4 mm. in width, separating the experimental from the control sections, was discarded. This was done to eliminate as far as possible any error which might arise from the diffusion of any of the injected substance. Macroscopic observations accompanied the respiration determinations. Also small sections of both control and experimental skin were reserved for histological study.

In preparation for the respiration experiments, the skin was washed in Ringer-phosphate-glucose solution containing 0.2

[9]Andervont, H. B., *U. S. Pub. Health Repts.*, 49 (1934): 620.

TABLE I

The Effect of Methylcholanthrene Injection on the Respiration of Mouse Skin

Experiment	Age of Mouse in Months	Sex	Time after Injection in Days	Control Qo_2	Experimental Qo_2	Per Cent Depression	Condition of Experimental Skin
1	3	♂	¾	1.100	.335	70	Evidence of lard at site of injection
2	3	♂	1	.048	.030	38	"
3	4	♂	4	.104	.042	60	"
4	4	♂	6	.140	.061	57	"
5	4	♂	11	.078	.060	23	"
6	4	♂	14	.093	.050	46	"
7	4	♂	18	.155	.107	31	"
8	3	♂	21	.133	.133	0	Keratic epithelium
9	4	♂	26	.141	.060	58	Lard encapsulated
10	3	♂	28	.215	.060	70	"
11	4	♂	31	.165	.059	65	"
12	4	♂	33	.379	.226	40	Evidence of hyperplasia
13	4	♂	45	.185	.053	71	Normal
14	6	♂	53	.208	.152	27	Tumor-like nodule near site of injection

per cent glucose. To eliminate error due to bacterial contamination the solution was sterilized by passing through a Berkefeld filter. All tissues, fatty or connective, were carefully removed from the skin sections, the respiration of which was to be studied. The skin was then rewashed in Ringer-phosphate-glucose solution and cut into small squares (approximately 1 mm.), thus exposing a large respiring surface. The control and the experimental tissues were each divided into two portions and placed in Warburg manometer flasks containing 0.2 cc. KOH and 3.1 cc. Ringer-phosphate-glucose solution. The equilibrium period was 15 minutes and the readings were taken at 60-minute intervals over a period of 120 minutes. The respiration was calculated according to the method of Warburg.

The Qo_2 values of the control and experimental skin are shown in Table I. The respiration measurements were begun 18 hours (¾ of a day) after the methylcholanthrene injections and continued through to the 53rd day after injection. Notwithstanding the variations in oxygen consumption of the individual mice, there is a definite respiratory depression of the injected area in every instance except in Experiment 8. Age difference, difference in genetic constitution, and variations in the general health of the animals are probably responsible for the variation in depression observed.

Another factor contributing to these variations is that shortly after the injection of the carcinogenic agent a small amount of the material is frequently lost through the puncture made by the needle. The average depression in respiration, due to the methylcholanthrene injections, was approximately 46 per cent.

To make certain that the lard itself had no effect upon the respiration of the skin, 3 mice were injected with lard alone. Table II gives a comparison between the injected and control areas of this experiment. It will be observed that the per-

TABLE II

The Effect of Lard Injection on the Respiration of Mouse Skin

Experiment	Age of Mouse in Months	Sex	Time after Injection in Days	Control Qo_2	Experimental Qo_2	Per Cent Change in Respiration
1	3	♀	¾	.178	.149	17 depression
2	3	♀	2	.282	.302	7 stimulation
3	9	♂	4	.121	.107	12 depression

centage variation obtained is but slightly above the allowed 10 per cent variation for experimental error.

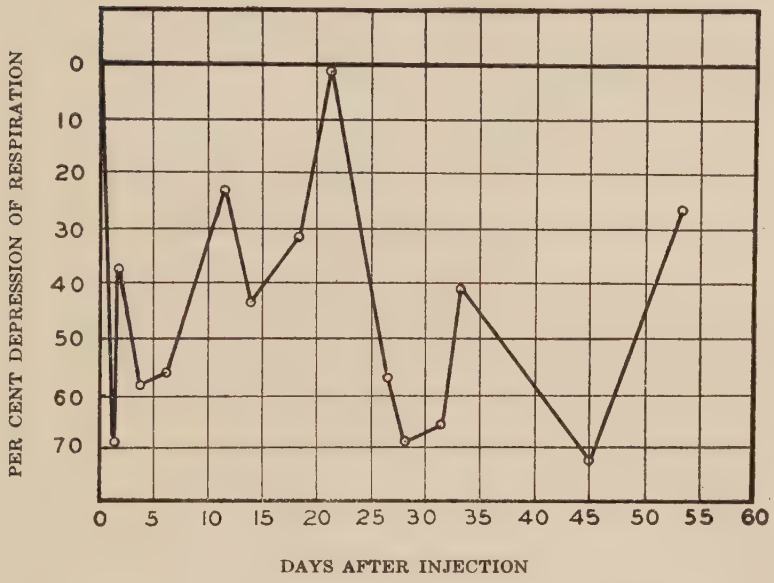
Several mice injected with methylcholanthrene, but not sacrificed for respiration determinations, developed tumors about 3 months after the treatment. These tumors had attained diameters of 2 cm. when the animals were sacrificed for histological studies. The histological observations revealed nothing unusual for this type of tumor.

CONCLUSIONS

The experimental evidence presented shows that, prior to cancerization, the tissue treated with methylcholanthrene suffers a marked depression in respiration. This change of respiration is effected almost immediately after the injection of the hydrocarbon and continues until tumor development.

It appears from these results that tumor tissue does not arise simultaneously with a deficiency in respiration since the depression in respiration is as high during the first few days after injection as it is toward the end of the period. Whether the decrease in respiration is the result of the death of a number of cells, caused by their inability to glycolyze, as suggested by Warburg, or that the respiratory mechanism of

Respiration of Mouse Skin Following Methylcholanthrene Injection



the cells is in some way impaired by the methylcholanthrene, has not been determined. Histological observations of the treated and untreated skin, though taken from insufficient material to warrant definite conclusions, show no appreciable difference between the number of dead and living cells, nor could an increase in mitotic figures be detected during this period.

Whatever may be the true picture of tumor etiology, a change in respiration appears to be definitely associated with it. This observation is further strengthened by the fact that respiration is high in immune animals (whether their immunity is natural or induced) and low in susceptible strains.

SUMMARY

1. The injection of methylcholanthrene depresses the respiration of the skin of mice near the site of injection.

2. The depressed respiration appears to continue until the onset of cancerization.

3. Normal respiration is not affected by the injections of lard, the agent in which the hydrocarbon was dissolved.

LITERATURE CITED

Andervont, H. B., *U. S. Pub. Health Repts.*, **49** (1934): 620.

Cook, E. S., *Chemical Products*, **1** (1939): 65; **2** (1939): 89.

Cook, E. S., Hart, Sr. M. J., and Joly, R. A., *Am. J. Cancer*, **35** (1939): 548.

Davis, J. E., *Can. Med. Assoc. J.*, **36** (1937): 27.

Dyadyuslia, G. F., *J. Med., Ukraine*, **7** (1937): 131.

Fardon, J. C., Brotzge, G., and Loeffler, Sr. M. K., This Issue, p. 61.

Fardon, J. C., and Sullivan, W. A., *Nature*, **143** (1939): 287.

Pourbaix, Y., *Compt. rend. soc. biol.*, **110** (1932): 1015; **112** (1933): 1222; **113** (1933): 930; **115** (1934): 1738.

Warburg, O., *J. Cancer Research*, **9** (1925): 148.

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THE RELATION OF INDUCED TUMOR RESISTANCE TO INCREASED TISSUE RESPIRATION*

By JOHN C. FARDON, REV. GUSTAVE C. BROTZGE AND SISTER M. KENNETH LOEFFLER, O.P.

THOUGH there has been some controversy among different investigators over the various kinds of tumors to which resistance could be elicited, it is generally accepted that resistance can be established to transplantable carcinoma through injections of various tissue extracts. Itami[1] has enumerated many tissues which he found could immunize against a transplantable carcinoma. Since placenta was used as the immunizing agent in our investigations we shall confine ourselves mainly to the behavior of this substance.

Higuchi[2] in 1912 found that placenta of mice produced a marked immunity toward both carcinoma and sarcoma. The activity of placenta was exceeded only by embryo skin and mammary tissue. He also found that dead tissues could not bring about this resistance, but rather induced a hypersusceptibility.

One of the authors of this paper[3] found such hypersusceptibility to mouse adenocarcinoma after an injection of the alcoholic precipitate of embryo tissue juice. Engel[4] found that the development of mouse carcinoma was inhibited by preparations containing the gonadotropic hormone obtained from either placenta or the hypophysis. Murphy and Sturm[5] observed that *desiccated* embryo skin and placenta exert a

*Research conducted under the Alex Meis Grant.

[1]Itami, S., *J. Cancer Research*, **4** (1919): 23.

[2]Higuchi, S., *5th Imp. Canc. Research, cf. Chem. Abst.*, **7** (1913): 642.

[3]Fardon, J. C., *Bull. Basic Sci. Research*, **5** (1933): 5.

[4]Engel, P., *Z. Krebsforsch.*, **41** (1935): 488.

[5]Murphy, J. B., and Sturm, E., *J. Exptl. Med.*, **60** (1934): 293.

definite retarding action on the growth of two transplantable mouse carcinomas, with no effect, however, on sarcoma. In some cases these investigators bathed the tumors in the extract before explantation. Using *fresh* placenta extract, they found no such immunity.

Prior to 1933 the contention generally accepted was that immunity to tumor growth could only be established by the introduction into the host of living cells of certain tissues. It was our observation[3] that immunity to mouse carcinoma could be induced by the injection of a cell-free fraction of embryo tissue juice. Recently Lewisohn[6] showed that by repeated injections of heterologous spleen extract (water extraction, adsorption with activated carbon, triple extraction with alcohol and elution) he was able to cause the complete disappearance of the mouse sarcoma No. 180 in 60 per cent of the treated animals.

Before considering the possible relation between tumor resistance and tissue respiration it might be well to give a brief resumé of the earlier work done in these and other laboratories with respiratory-stimulating factors obtained from injured cells.

The first evidence in these laboratories that the respiratory mechanism of cells could be stimulated was obtained through the irradiation of yeast cells by ultraviolet.[7] Later Cook, Kreke and Nutini[8] demonstrated that certain factors were present in yeast which could stimulate the respiration of rat skin and liver. The experiments of Ruddy[9] showed that the cell-free fraction of minced rat spleen, liver and kidney tissue greatly

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[6]Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

[7]Fardon, J. C., Carroll, Sr. M. J., and Ruddy, Sr. M. V., THESE STUDIES, **1** (1937): 17.

[8]Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

[9]Ruddy, Sr. M. V., THESE STUDIES, **2** (1938): 21.

stimulated the respiration of yeast cells. Büngeler,[10] working on the principle that animal spleens must contain a factor harmful to tumors, since tumors of the spleen are exceedingly rare, found that extracts of spleen enhanced the respiration and depressed the glycolysis of tumor tissue. He found the active agent in the extract to be water-soluble, not combined with protein or lipoids, not destroyed at 100° C., and dialysable. Lymph-nodes, thymus, lung, heart and testes were also found to be effective, but not in the same degree. Carroll[11] also observed that spleen extract stimulated the respiration of tumor tissue. Recent experiments in these laboratories[12] indicate clearly that the respiration of liver, sarcoma and carcinoma can be stimulated by extracts of heart, liver, kidney, thyroid and testes.

Since it has been found that most tissue extracts have the property to stimulate the respiration of both normal and malignant tissue, and since many of these extracts also appear to be instrumental in eliciting resistance to tumor growth, it occurred to us that a relationship might exist between cellular respiration and induced tumor immunity. The present experiments were conducted to determine if the so-called immunizing fraction is really a respiration-stimulating agent and if tumor resistance can only be realized when the general cellular respiration is increased. It has already been shown by Davis[13] that *natural* resistance or susceptibility to cancer is dependent upon the respiration of the normal tissues of the animal.

Experimental

The placenta from 12 pregnant mice were removed under aseptic conditions and suspended in 6 cc. of Drew's solution.

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[10]Büngeler, W., *Frankfurt. Z. Path.*, **43** (1932): 409.

[11]Carroll, Sr. M. J., *Nature*, **143** (1939): 684.

[12]Unpublished results.

[13]Davis, J. E., *Can. Med. Assoc. J.*, **36** (1937): 27.

TABLE I

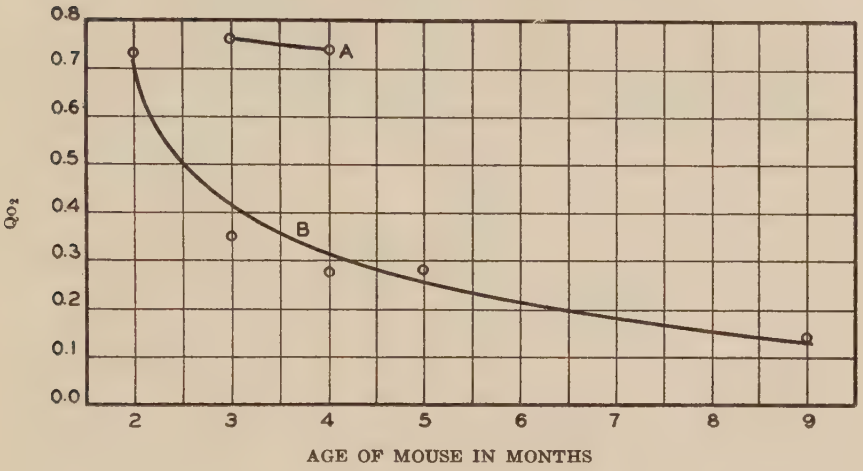
Difference in Qo_2 between Skin over Site of Injection and Control Skin.

Experiment	Day after Injection	Age of Mouse in Months	Sex	Control Skin Average Qo_2	Experimental Skin Average Qo_2
1	2	4	♂	0.657	0.566
2	3	4	♂	0.456	1.148
3	4	3	♂	0.956	0.677
4	6	3	♂	0.834	0.491
5	7	3	♂	0.620	0.285
6	9	3	♂	0.759	0.535
7	10	4	♂	1.184	0.725
8	11	3	♀	0.655	0.699
9	12	4	♀	0.668	0.361
Average				0.754	0.610

Average depression of respiration of experimental skin = 23 per cent.

Into each of 12 mice, 0.5 cc. of this suspension was injected subcutaneously. One of these mice was sacrificed each day to determine the respiration of its skin tissue. About 1 sq. cm. of skin was carefully dissected from the site of the injection and an equal portion removed from a similar location on the other side of the animal. Hair, connective tissue and fat were carefully removed from all pieces of skin. With the aid of a pair of scissors the control and experimental skins were cut into cubes about 1 mm. square. Both control and experimental tissues were divided into two equal portions and introduced into the Warburg-Barcroft respirometer flasks. The usual technique was followed, with an equilibrium period of 15 minutes and a respiration period of 2 hours. The tissue fragments were washed, dried overnight in a drying oven and weighed. Table I shows the results. Three of the 12 mice died during the experiment.

FIG. 1—*Normal Respiration of Mouse Skin as a Function of Age (B) and after Placenta Injection (A)*



It will be observed that on the average the skin from the site of injection gave a lower Qo_2 than the control skin. The average depression of respiration of the experimental skin was about 23 per cent. Since there was no evidence of inflammation or infection at the site of injection, the resultant depressed respiration of this area of skin could only be considered as due to some sort of local tissue reaction.

The interesting and important fact which is brought to light in this experiment is that the Qo_2 of both the experimental and the control skin is considerably higher than that usually found in normal stock mice of the same age. Table II, for example, gives the Qo_2 of skin tissue from a group of 24 mice. The average of the mice between the ages of 3 to 4 months in this control group gives a skin Qo_2 of 0.317. If this is compared to the Qo_2 of the experimental series in Table I, we observe an increase of about 92 per cent in the latter. The control series of Table I shows an increase of 137 per cent. Curve B of Fig. 2 shows the fall in respiration

TABLE II

Qo_2 of Skin of a Series of Normal Control Mice.

Experiment	Age of Mouse in Months	Sex	Skin Qo_2	Experiment	Age of Mouse in Months	Sex	Skin Qo_2
1	4	♀	0.170	13	4	♂	0.187
2	4	♂	0.154	14	4	♂	0.156
3	3	♀	0.357	15	4	♂	0.279
4	3	♀	0.562	16	5	♂	0.416
5	4	♂	0.370	17	5	♂	0.207
6	4	♂	0.757	18	5	♂	0.276
7	4	♂	0.330	19	2	♂	0.686
8	3	♂	0.430	20	2	♂	1.307
9	4	♂	0.282	21	2	♂	0.299
10	3	♂	0.266	22	2	♀	0.620
11	3	♂	0.148	23	2	♀	0.762
12	4	♂	0.310	24	9	♂	0.147

Total average skin $Qo_2 = 0.317$ (only includes mice 3 to 4 months old).

of the skin with increasing age, while Curve A gives the elevated respiration of mice between 3 and 4 months of age as a result of the placenta injection. Thus it can be readily observed that the general respiration of at least the skin is considerably increased following the injection of the placental suspension. Under these conditions it has been shown repeatedly by ourselves and others that immunity to certain types of tumors is induced.

It might be of interest at this time to refer to the work of Strong,[14] who, using the "A" strain of inbred mice, found that only 1 per cent of female mice between 3 and 4 months of age developed spontaneous carcinoma of the breast, while those about 9 months old showed a tumor incidence of 9 per cent. The maximum (13 per cent) occurred after some 12 months. Though one hesitates, in view of the two different problems,

[14]Strong, L. C., *Am. J. Cancer*, **30** (1937): 527.

to correlate the results of Strong with the authors' observations, it is interesting to note how tumor incidence within certain limits increases with age while the respiration of at least the skin decreases with age. Of greater interest and significance, of course, is the fact that the respiration of 3 to 4 month-old skin can be stimulated to the value ordinarily obtained for mice 2 months old.

Conclusions

The experimental results show that an injection of placental suspension into mice definitely stimulates the oxygen consumption of the skin.

Placenta, which is known to be a good immunizing agent, has been shown to increase the respiration of mouse epithelium. Since many investigators have shown that a decreased respiration is associated with tumor development, and others that an increased respiration is characteristic of the tissues of animals naturally resistant to certain tumors, it seems plausible that tumor-immunizing agents act by increasing the respiration of the normal tissues.

Because it is generally held that tumor immunity reaches a maximum about 7 days after injection of the immunizing agent, it was expected that the respiration should follow this same gradient. It was found, however, that the respiration was enhanced shortly after the injection and remained constant throughout the duration of the experiment. As a result of this lack of correlation it must be assumed either that the lag period for immunization has not been accurately estimated for placenta, or that increased respiration is not directly responsible for immunization but merely a phenomenon accompanying immunization. When one considers, the high tumor incidence in old animals as compared to young animals, and that in the former the respiration is much lower than in the latter, it is apparent that a definite relationship exists

between tumor immunity, metabolism, and age. The significance of this relationship remains to be investigated.

Summary

1. After an injection of placental suspension the general respiration of mouse skin is considerably increased.

2. Though the respiration of the skin at the site of the injection shows a definite depression compared to the control portion of skin from the same mouse, the oxygen consumption of this affected area is greater than that found in a normal control mouse.

3. The increased respiration of the skin brought about by the injection of the placental suspension appears to be established shortly after the injection, and remains of the same average magnitude from the second to the twelfth day after the injection.

4. A possible relationship between induced tumor immunity and increased tissue respiration is suggested.

LITERATURE CITED

Büngeler, W., *Frankfurt. Z. Path.*, **43** (1932): 409.

Carroll, Sr. M. J., *Nature*, **143** (1939): 684.

Cook, E. S., Kreke, C. W., and Nutini, L. G., These Studies, **2** (1938): 23.

Davis, J. E., *Can. Med. Assoc. J.*, **36** (1937): 27.

Engel, P., *Z. Krebsforsch.*, **41** (1935): 488.

Fardon, J. C., *Bull. Basic Sci. Research*, **5** (1933): 5.

Fardon, J. C., Carroll, Sr. M. J., and Ruddy, Sr. M. V., These Studies, **1** (1937): 17.

Higuchi, S., *5th Imp. Canc. Research, cf. Chem. Abst.*, **7** (1913): 642.

Itami, S., *J. Cancer Research*, **4** (1919): 23.

Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

Murphy, J. B., and Sturm, E., *J. Exptl. Med.*, **60** (1934): 293.

Ruddy, Sr. M. V., These Studies, **2** (1938): 21.

Strong, L. C., *Am. J. Cancer*, **30** (1937): 527.

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March 17, 1940

THE ROLE OF HOMOLOGOUS AND HETEROLOGOUS TISSUE EXTRACTS IN THE ESTABLISHMENT OF THE RESISTANT STATE AGAINST CARCINOMA*

By JOHN C. FARDON, REV. GUSTAVE C. BROTZGE, and SISTER M. KENNETH LOEFFLER, O.P.

Since Ehrlich[1] in 1906 succeeded in rendering animals resistant to tumors by inoculation with tumor tissue of low virulence, others have continued these investigations, using both tumor and normal tissues to elicit the resistant state. Bashford[2] found that after the spontaneous absorption of the Jensen's mouse tumor, resistance continued for at least six months. In a review of experimental work, Woglom[3] pointed out that Michaelis found that cancer cells killed by heat or chloroform lost their power to produce resistance. Flexner and Jobling[4] had obtained similar results. Haaland[5] showed that complete disintegration of tumor and normal cells by any of a variety of physical agents robs them of the power to induce resistance.

Subjecting mouse spleens to crushing, freezing and thawing, and inoculating the material into splenectomized mice, Woglom[3] found no resistance induced to mouse tumor No. 63. Higuchi[6] found that placenta of mice produced a marked immunity to carcinoma and sarcoma, which was exceeded in activity only by embryonal skin and mammary tissue. He

*Research conducted under the Alex Meis Grant.

[1]Ehrlich, P., *Z. Ärztl. Fortbild.*, **111** (1906): 211; *Arb. Staatsinst. Exptl. Therap.*, **1** (1906): 90.

[2]Bashford, E. F., *Brit. Med. J.*, **2** (1906): 209.

[3]Woglom, W. H., *J. Exptl. Med.*, **12** (1910): 29.

[4]Flexner, S., and Jobling, J. W., *Proc. Soc. Exptl. Biol. Med.*, **5** (1907): 5

[5]Haaland (unpublished) quoted by Bashford, *Med. Record*, **76** (1909): 381.

[6]Higuchi, S., Fifth Sci. Report on Investigation of Imp. Cancer Research.

also found that rat tissue apparently produced a weak immunity to mouse carcinoma, but the results were inconsistent and slight. Dead tissues were not found to produce immunity, but were rather inclined to develop conditions of hypersusceptibility. Itami[7] demonstrated that resistance to carcinoma could be induced by injection of some but not all of the derivatives of the three germinal layers. In 1926 one of the authors[8] showed that immunity could be produced against the adenocarcinoma No. 63 by injection of either embryo or spleen cell-free extracts. Prior to these investigations, it was generally accepted by most workers that suspensions of living cells were necessary to establish resistance against tumors. Later, Büngeler,[9] depositing spleen concomitantly with tumor inoculation, found delayed growth of the Ehrlich mouse chondroma and carcinoma. He found the active agent to be water-soluble, not combined with proteins and lipoids, dialysable, and stable at 100° C.

Murphy[10] in 1934 found that autografts, after being in contact with extracts of desiccated embryo skin or placenta for short intervals, either failed to grow, or, in the majority of cases had subsequent growth retarded. Though Murphy observed that fresh extracts of placenta were without effect, it will be recalled that Higuchi found these extracts to produce a marked immunity to carcinoma and sarcoma. Dittmar,[11] giving preliminary treatment with extracts of Ehrlich mouse carcinoma, found some delay in the growth of that tumor, or a diminished number of takes. Dittmar (at variance with the author's observations[8]) found this extract to be more active than those obtained from mouse spleen. Using chick embryo

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[7]Itami, S., *J. Cancer Research*, **4** (1919): 23.

[8]Fardon, J. C., *Bull. Basic Sci. Research*, **5** (1933): 5.

[9]Büngeler, W., *Frankfurt Z. Path.*, **43** (1932): 409.

[10]Murphy, J., *J. Exptl. Med.*, **60** (1934): 305.

[11]Dittmar, C., *Am. J. Cancer*, **31** (1937): 109 (abs.).

skin, Gardner and Hyde[12] were able to immunize to some extent against the sarcoma, but not against the carcinoma. Though MacFadyen, Sturm and Murphy[13] found that mammary tissue of pregnant rabbits and of a cow caused inhibition of the Bashford carcinoma, they brought the tumor in contact with the extracts before inoculation into mice. The inhibition of tumor growth thus obtained with heterologous tissues appears to be a direct effect of the pulp rather than one of immunization. It is hardly necessary to continue with further citations from the abundance of available literature on the subject of cancer resistance. It is of considerable interest, however, to call to mind that in spite of the many inconsistencies and contradictions encountered in this phase of the cancer problem, resistance to tumors can definitely be induced artificially in most, if not all, experimental animals.

It is the purpose of this paper to present a series of preliminary experiments on tumor resistance and on the efficacy of homologous and heterologous tissues in establishing that resistant state. The isolation of the active principle is also attempted.

EXPERIMENTAL

Throughout the immunity experiments conducted in these laboratories, the mammary carcinoma No. 15091a, obtained from the Jackson Memorial Laboratory, was used. Transplants of this tumor were made into the ABC strain of mice, which were obtained from the same laboratory. This strain of mice was used only for maintaining the tumor stock.

Stock mice (unknown genetic constitution, obtained from a local dealer) were used for the preparation of the immunizing agents and for subsequent resistance tests. The No. 15091a

[12]Gardner, R. E., and Hyde, R. R., *Am. J. Cancer*, **16** (1932): 15 (abs.).

[13]MacFadyen, D. A., Sturm, E., and Murphy, J. B., *J. Exptl. Med.*, **70** (1939): 475.

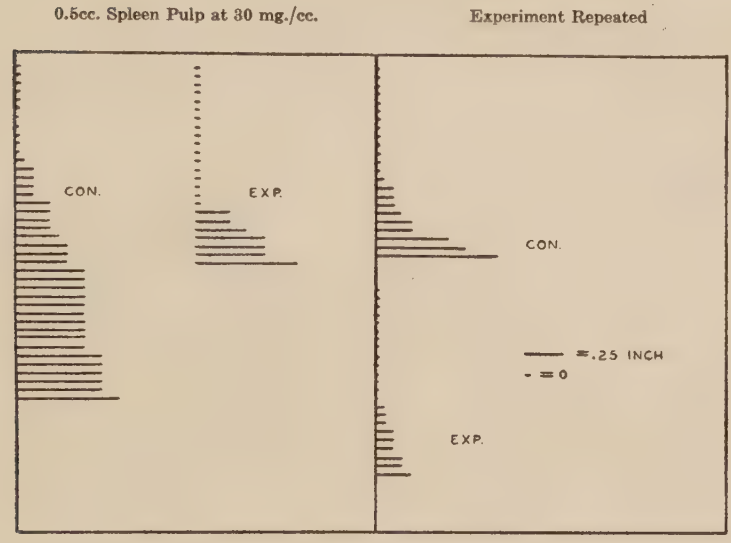
tumor usually gave about 75 to 80 per cent takes in the control animals used by the authors and their associates. Tumor inoculations were usually made as follows: tumors measuring about ¾-inch in diameter and comparatively free from necrotic material, were removed from the animals under aseptic conditions. After removal, the tumor was cut into small fragments and pressed through a fine wire mesh, and 10 cc. of Drew's solution added. With a tuberculin syringe, 0.25 cc. of the tumor suspension was inoculated subcutaneously into the abdominal region of the animals. Measurements of tumor size in the control and the experimental mice were begun 7 to 10 days after the inoculation. The final measurement taken after about 3 weeks was recorded and expressed in the charts.

It might be mentioned that both spleen and embryo tissue preparations have been successfully used in these laboratories for immunizing against the mouse mammary carcinoma No. 63. Extracts from these two preparations have always shown good immunizing properties. It was thought advisable, however, to repeat the experiments for this particular discussion.

The immunizing property of spleen pulp was tested. Spleens were removed from mice under aseptic conditions, cut into small pieces, and pressed through a fine wire mesh. About 1 cc. of Drew's solution was added per spleen. A group of 40 stock mice were then injected with 0.5 cc. of this pulped material. The suspension contained about 30 mg. of dried material per cc. Seven days after the injection of the spleen pulp, the animals received a tumor inoculation by the method described above. An additional 40 mice were also inoculated with the tumor and served as controls.

The chart in Fig. 1 illustrates the relative immunity obtained with the spleen pulp. Of the 40 animals in the experimental group, 16 died. However, of the remaining mice, 29 per cent developed tumors, while in the control group 70 per cent takes

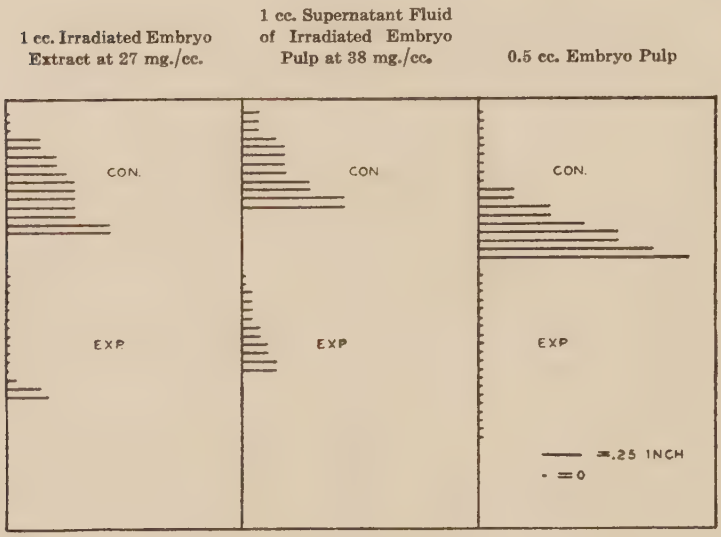
FIG. 1—*Resistance to Carcinoma in Mice Induced by Spleen Pulp*



were obtained. Tumors which measured less than $\frac{1}{8}$ inch in diameter after 3 weeks were considered as negative. In repeating the experiment, using 24 mice for each group, it was found that tumors developed in 25 per cent of the experimental animals and in 39 per cent of the controls. Though the immunity was not as marked as in the first experiment, the tumors in the control animals were relatively larger than in the experimental. The tumor used in this experiment obviously had lost some of its virulence.

The effectiveness of mouse embryo tissue was tested in the second series of experiments. Embryo pulp was prepared in much the same manner as the spleen pulp in the foregoing experiments and the same injection procedure was used. As shown in Fig. 2, in the control animals 56 per cent developed tumors, while none developed in the experimentals.

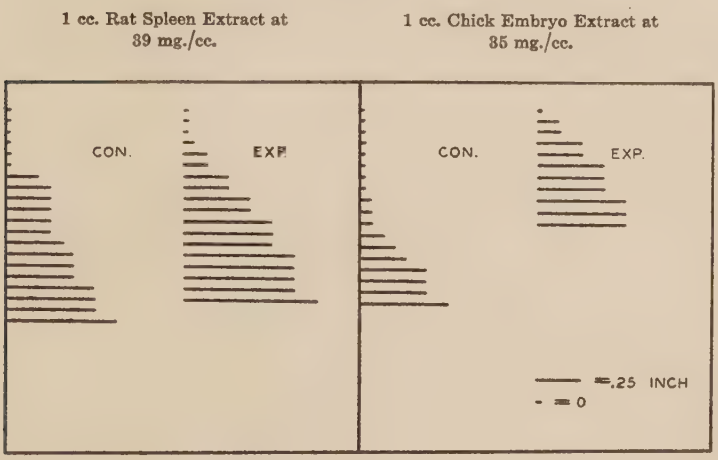
FIG. 2—*Resistance to Carcinoma Induced by Embryo Pulp and Extracts*



In an attempt to liberate more of the immunizing principle from the pulped embryo tissue, the tissue was spread in a shallow dish and irradiated with ultraviolet light for a period of 30 minutes. Since Loofbourow *et al.*,[14] of these laboratories, had shown that cell-free extracts of mouse or rat embryo pulp irradiated with ultraviolet light produced, when repeatedly injected into mice, tumor-like growths which were more numerous and larger than those obtained with non-irradiated extract, it was thought by the authors that some such procedure might produce a more active immunizing agent. After irradiation of the embryo pulp, the material was centrifuged for 15 minutes, the supernatant fluid pipetted off, and 1.0 cc. injected per mouse. The controls for this experi-

[14]Loofbourow, J. R., Cueto, A. A., Whalen, D., and Lane, Sr. M. M., *Nature*, 144 (1939): 939.

FIG. 3—*Resistance to Carcinoma in Mice Induced by Spleen Extract and Chick Embryo Extract*

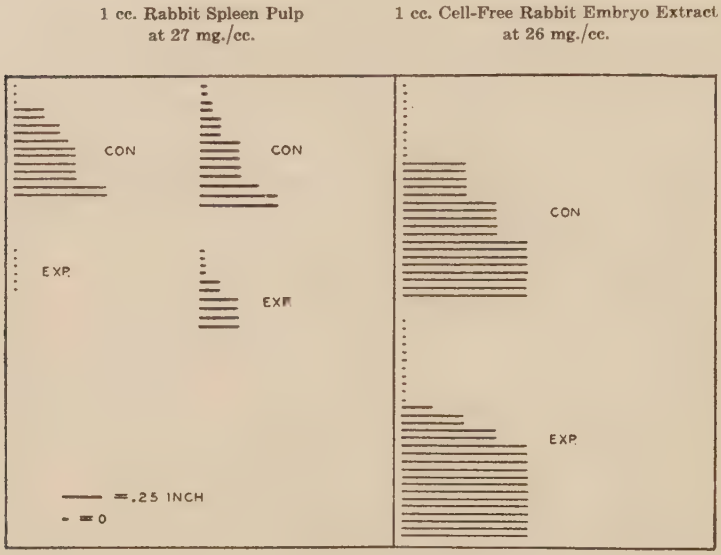


ment produced 100 per cent tumor takes, the experimentals 50 per cent. It will be noted from the graph that the controls produced much larger tumors.

A third experiment tested the effect of irradiation of the supernatant fluid only. The question arose as to whether irradiation would result in activation of the fluid or, contrariwise, have a tendency to destroy the immunizing principle. The experiment indicated no destructive action due to irradiation. The controls in this instance gave 80 per cent takes as compared with 13 per cent takes among the experimentals. From present experimental work it seems that the immunizing fraction is more or less indifferent to radiations. Incidentally, the experiment demonstrates the feasibility of using ultraviolet radiations for the sterilization of the immunizing fraction.

It is obvious that, should induced immunity to cancer eventually be proved practical for human cases, it will be necessary to determine whether homologous tissues must be

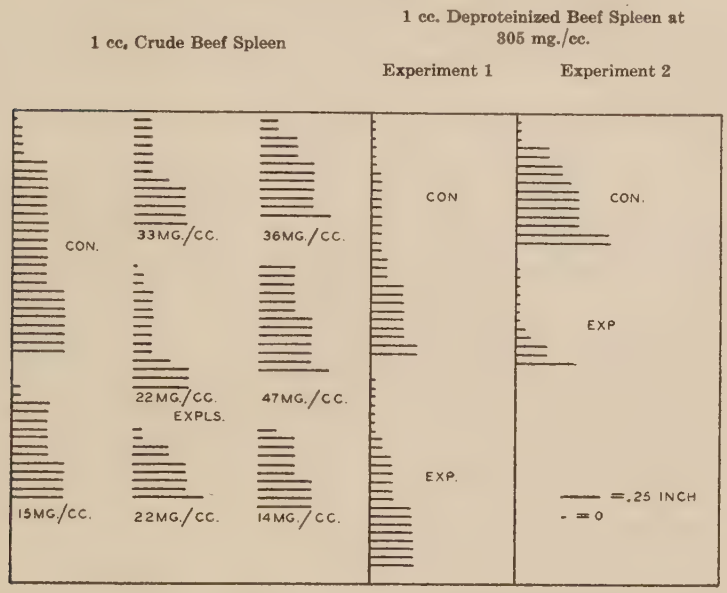
FIG. 4—*Resistance to Carcinoma in Mice Induced by Rabbit Spleen Pulp and Rabbit Embryo Extract*



used for the preparation of the immunizing fraction, or whether extracts prepared from heterologous tissues will be equally effective without producing a serious reaction in the host. The practical advantages of the use of heterologous tissues are, of course, so obvious as to require no further discussion. The heterologous tissues tested for their immunizing property were rat spleen, chick embryos, rabbit spleen, rabbit embryos, and beef spleen. The rat spleens were minced in the same manner as were the mouse spleens. After centrifugation of the pulp, the supernatant fluid was used for injection. The results are shown in Fig. 3. The slightly higher percentage of takes among the experimental animals is within experimental error.

The results of the use of chick embryo extract proved to be more discouraging than the results obtained with rat spleen.

FIG. 5—*Resistance to Carcinoma in Mice Induced by Crude Beef Spleen and Deproteinized Beef Spleen*



Eight-day-old chick embryos were used for the preparation of the extract. Although 7 animals of the experimental group died, it is quite evident that this extract did not protect the mice against the tumor inoculation. In view of the rather unusually low percentage of takes among the controls, it appears that, if anything, the chick embryo extract stimulated the growth of the tumor.

Rabbit embryo extract also failed to elicit resistance against the mouse carcinoma. As shown in Fig. 4, about an equal number of takes were obtained in both the control and the experimental animals. Rabbit spleen pulp seemed to produce some degree of resistance, but the experiment was inconclusive since 60 per cent of the experimental animals died before the potential tumors could be established to the extent of allowing

measurements. The experiment with rabbit spleen was repeated and, though some degree of immunity was obtained, it was not very marked. The controls showed about 73 per cent takes as compared with 67 per cent in the experimental group.

The effectiveness of beef spleen as an immunizing agent was also found to be questionable. Various concentrations of beef spleen pulp, ranging from 14 mg./cc. to 47 mg./cc., were prepared and injected into mice. The results of these experiments are illustrated in Fig. 5. The immunizing agent is listed as "crude beef spleen." Judging from tumor size, some slight degree of immunity seems to have been obtained with concentrations of 22 and 33 mg./cc.; however, concentrations lower and higher than these values seemed to have no effect on the growth of the tumors. One must conclude, from the average of these experiments, that the crude beef spleen does not induce the resistant state in mice.

With the failure of these preliminary experiments, deproteinized beef spleen was prepared after the manner of Lewisohn.[15] It has been shown by Lewisohn that subcutaneous injections of concentrated beef spleen extracts into established sarcomata caused regression and final disappearance in 60 per cent of the cases. Control mice showed spontaneous regression in 8 per cent of the cases. Subcutaneous injections of 0.5 cc. of concentrated spleen extract (100 mg. of fresh spleen in 1 cc.) were found to be the effective dose. Smaller doses of the extract were found by him to have a stimulating effect upon the tumors. In the authors' experiments, using the deproteinized beef spleen fraction, an attempt was made to immunize rather than destroy an established tumor. The extract was prepared in a concentration of 305 mg./cc. One cubic centimeter of this extract was injected subcutaneously

[15]Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

into the experimental mice. No immunity could be demonstrated 3 to 4 weeks after the tumor implantation. The experiment was repeated, using the same concentration and dosage, with the result that the control mice gave 80 per cent takes against the experimental 33 per cent (see Fig. 5).

CONCLUSIONS

That immunity can be induced to mouse carcinoma by the injection of mouse spleen and embryo extract has again been verified. In no instance, however, have comparable results been obtained with the use of heterologous tissues. It is concluded, therefore, from the above investigations that realization of the resistant state is associated with tissue specificity. These experiments confirm the work of Higuchi,[6] who found that rat tissues produced only a weak immunity to mouse carcinoma.

Experiments are under way to isolate and concentrate the active immunizing fraction from the homologous tissues tested.

SUMMARY

1. Mouse spleen and mouse embryo extracts were found to immunize mice against the transplantable mammary carcinoma No. 15091a.

2. Irradiation with ultraviolet light neither stimulated nor inhibited the active immunizing principle in mouse embryo pulp or extract.

3. Rat spleen extract, chick embryo extract, rabbit spleen pulp, embryo extract, crude beef spleen, and deproteinized beef spleen extract failed to induce a resistant state against carcinoma in mice, comparable to that elicited by the homologous tissue pulps and extracts.

LITERATURE CITED

Bashford, E. F., *Brit. Med. J.*, **2** (1906): 209.

Büngeler, W., *Frankfurt Z. Path.*, **43** (1932): 409.

Dittmar, C., *Am. J. Cancer*, **31** (1937): 109 (abs.).

Ehrlich, P., *Z. Ärztl. Fortbild.*, **111** (1906): 211; *Arb. Staatsinst. Exptl. Therap.*, **1** (1906): 90.

Fardon, J. C., *Bull. Basic Sci. Research*, **5** (1933): 5.

Flexner, S., and Jobling, J. W., *Proc. Soc. Exptl. Biol. Med.*, **5** (1907): 5.

Gardner, R. E., and Hyde, R. R., *Am. J. Cancer*, **16** (1932): 15 (abs.).

Haaland (unpublished) quoted by Bashford, *Med. Record*, **76** (1909): 381.

Higuchi, S., Fifth Sci. Report on Investigation of Imp. Cancer Research.

Itami, S., *J. Cancer Research*, **4** (1919): 23.

Lewisohn, R., *Surg. Gynecol. Obstet.*, **66** (1938): 563.

Loofbourow, J. R., Cueto, A. A., Whalen, D., and Lane, Sr. M. M., *Nature*, **144** (1939): 939.

MacFadyen, D. A., Sturm, E., and Murphy, J. B., *J. Exptl. Med.*, **70** (1939): 475.

Murphy, J., *J. Exptl. Med.*, **60** (1934): 305.

Woglom, W. H., *J. Exptl. Med.*, **12** (1910): 29.

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STUDIES IN POLLEN TUBE CULTURE*

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*Research conducted under the Sir Charles F. Williams Grant.

I. Introduction

While the growth of pollen tubes may be studied in the living state or in fixed preparations, their physiology is best studied in the living state. The tubes may be grown on the stigma of flowers or in artificial media. The authors studied the growth of pollen tubes in the living state on artificial substratum. The purpose of this study is the development of a practical method of pollen tube culture. The present work confines itself to the culturing of pollen tubes under normal and modified conditions. Elsewhere some of the growth phenomena are discussed.[1]

A suitable test object was requested by several investigators of these laboratories, who are interested in growth-promoting substances, their isolation, and the physiological effects produced by them.[2] The authors demonstrated that cultured pollen tubes respond readily to factors in the environment in general and to biochemical regulators in the medium in particular.[1] Such cultured pollen tubes are therefore favorable test objects for growth-promoting substances. The present study was undertaken in order to gain better acquaintance with the normal growth of pollen tubes and the phenomena naturally accompanying it, before proceeding to test the effectiveness of biochemical regulators upon the test object.

In this work the authors studied the nature and the required quantity of the medium to obtain favorable growth; the various kinds of pollen which are best suited for this purpose; the occurrence of growth-promoting substances that are naturally associated with the floral organs; the conditions that affect the degree of germination; and, finally, the rate and total growth of the tubes under favorable circumstances.

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[1]Beck, W. A., and Joly, R. A., *Trans. Am. Microscop. Soc.*, **60** (1941): 149.

[2]Loofbourow, J. R., and Dwyer, Sr. C. M., *Studies Inst. Divi Thomae*, **2** (1939): 155. Morgan, Sr. M. N., *ibid.*, **2** (1939): 137. Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M., *ibid.*, **1** (1937): 163.

The effect of the bios factors, which might be expected to influence the division of the generative cell—a form of cell proliferation—is not treated exhaustively here, and the influence of the auxin factors which effect tube growth—a form of cell enlargement—is treated elsewhere.[1]

The tube nucleus is never visible in the living state,[3] therefore its study is impossible here. Some attention was given to the division of the generative cell and the phases that precede the division, because some important cytological problems are involved in the process, such as the movement of the cell, its relation to the cyclosis of the vegetative or tube plasm, the activity of the vegetative plasm immediately surrounding the generative cell, and the formation of the restitution nucleus. These facts should be known so that they may be recognized as normal in cultured pollen tubes.

II. Historical

A. *Nature of Pollen.* Camerarius[4] in 1694 was the first to demonstrate satisfactorily sexuality in the higher plants and recognized that pollen served as the male element. Up to this time pollen, the yellow dust produced by the anthers of the flowers of seed-bearing plants, was the object of a great many queer speculations and theories.[5]

More than a century elapsed before any definite idea was obtained regarding the manner in which the pollen functions in fecundation. The pollen tube was discovered in 1824 by Amici[6] while examining the papillary cells of the stigma of *Portulaca*, noting that the "hairs" terminated in pollen grains. In 1830 he traced the tubes through the tissues of the style into the ovary and showed that the tube is an organism and

[3]Trankowsky, D. A., *Planta*, 12 (1931): 1.

[4]Camerarius, R. J., De Sexu Plantarum, 1694.

[5]Wodehouse, R. P., Pollen Grains, McGraw-Hill, New York, 1935.

that it passes selectively to one ovule.[6] Hofmeister[7] clearly demonstrated that the embryo arises, not from the end of the pollen tube as Schleiden[8] and Schacht[9] thought, but from an egg contained in the ovule.

The real significance of the pollen tube was clearly demonstrated by Strasburger[10] when he showed that fertilization is essentially the union of the egg nucleus with the sperm nucleus brought in by the pollen tube.

B. *Growth-Promoting Substances.* As early as 1834 Fritzsche[11] describing the external morphology of the pollen grain, recognized a substance on the surface of the grain, which he called pollenin. It has been known for a long time that masses of pollen will cause growth and swelling on fruits. Fitting[12] proved that certain postfloration phenomena are due to the presence of pollen grains on the gynostemium. Some types of pollen require extracts of the gynostemium in the culture media to induce sprouting. Brink[13] successfully improved growth *in vitro* by the addition of traces of sterile yeast to the culture medium. He also found that the addition of other plant extracts improves growth, and suggested that the growth-promoting substance or substances may be protein derivatives or may be of a vitamin nature.

Kuhn[14] and the present authors proved that the pollen itself contains a substance of a growth-promoting nature. Kuhn

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[6]Amici, G. B., *Ann. sci. nat. Botan.*, I, 2 (1824): 41; I, 21 (1830): 329.

[7]Hofmeister, W., Die Entsehung des Embryo der Phanerogamen, Leipsig, 1849.

[8]Schleiden, M. J., *Wiegman's Arch.*, 1 (1837): 289.

[9]Schacht, H., *Ann. sci. nat. Botan.*, III, 15 (1851): 80. *Jahrb. wiss. Botan.*, 1 (1858): 193.

[10]Strasburger, E., Neue Untersuchungen über den Befruchtungsvorgang bei den Phanerogamen als Grundlage für eine Theorie der Zeugung, Jena, 1884.

[11]Fritzsche, K. J., *Poggendorf's Ann. Physik Chemie*, 32 (1844): 481.

[12]Fitting, H., *Z. Botan.*, 1 (1909): 1; 2 (1910): 225.

[13]Brink, R. A., *Am. J. Botan.*, 11 (1924): 283, 351, 417.

[14]Kuhn, E., *Planta*, 27 (1937): 305.

calls it "Keimungs fördernde Substanzen." The authors[15] working with liliaceous pollen also observed that sprouting takes place initially in the innermost regions of a colony, and indicated that the oil which covers the outside of the pollen grain is of a growth-promoting nature or at least is the vehicle of a growth-promoting substance. The authors suggest that it is of an auxin nature and correlate the phenomenon of bursting of tubes with the growth promoter. Thimann and Went[16] proved that auxin is present in the pollen of a great variety of flowers. Beck and Donnelly[17] were able to produce cell enlargement in the hypocotyl of Helianthus with the natural auxin from pollen.

C. *Artificial Culture.* In 1834, von Mohl[18] observed that the pollen of some species would form tubes readily in moist air and water but van Tieghem[19] is generally accredited with the first study of artificial germination of pollen. Since their time numerous investigators have successfully cultivated pollen *in vitro.* It was noted early that many pollens germinated over a wide range of concentration of sugar. A small amount of agar or gelatin added to the germinating medium improved germination with many species according to Mangin.[20] Other investigators, Molisch[21] and Lidfross,[22] reported that stigmas exert a strong stimulatory action when added to the medium. Molisch and Lidfross also noted chemotropic response of the pollen tubes of some species to sugar, diatase preparations,

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[15]Beck, W. A., and Joly, R. A., *Nature,* **145** (1940): 226.

[16]Thimann, K. V., and Went, F. W., Phytohormones, MacMillan Co., New York, 1937.

[17]Beck, W. A., and Donnelly, Sr. M. W., *Studies Inst. Divi Thomae,* **2** (1939): 179.

[18]Mohl, H. von, Beitrage zur Anatomie und Physiologie, Berlin, 1834.

[19]Tieghem, P. van, *Ann. sci. nat. Botan.,* V, **12** (1869): 312.

[20]Mangin, L., *Soc. Botan. France Bul.,* **33** (1886): 337, 512.

[21]Molisch, H., *Sitzungsber. Wien Akad. wiss. Math. Naturw.,* **102** (1893): 423.

[22]Lidfross, B., *Jahrb. wiss. Botan.,* **29** (1896): 1.

and egg albumin. Tokugawa[23] noted that the addition of some protein materials to pollen cultures often more than doubled the growth. Brink[13] discovered that yeast extracts increased growth as much as 142 per cent. Kuhn[14] showed that the amount of medium employed in a given culture influenced the growth of the tubes. This was confirmed by the authors,[1] who proved that the extract from the stigma produced the same effect as the whole tissue section, and adduced reasons to show that the biochemical regulator which is involved is rather of a growth-promoting nature than of a chemotropic one.

III. Theoretical

Morphology and Cytology of Pollen. The pollen grain is homologous with the microspores of vascular cryptogams. It is therefore a true spore, producing a gametophyte.

The pollen grain is a member of a tetrad and develops within the anther. The pollen mother-cell (microsporocyte) is its origin. A mucilaginous fluid which completely surrounds the pollen mother-cell is probably the origin of the growth-promoting substance which is found on the pollen grain covering. It is during the formation of the tetrad that reduction division occurs. The gametophyte which is artificially cultured is therefore haploid.

The pollen grain includes within itself the two members of the divided microspore nucleus, and around one of them a membrane cuts a generative cell, the outer nucleus and the remaining cytoplasm constituting the tube cell. The tube nucleus generally moves into the tip of the pollen tube and the generative nucleus follows. In artificial culture the length of the tubes may be related to the species. It may be assumed for a given species that in normal growth on the stigma the length of the tube is determined by the length of the style.

[23]Tokugawa, Y., *J. Coll. Sci. Imp. Univ. Tokyo*, 35 (1914): 1.

Naturally the grain and the conducting tissue must contain sufficient nutrient and growth-promoting substances to make this possible. The rate of growth varies considerably and is determined both by environmental factors and the genetic constitution of the pollen and the stylar tissue. Normally the tube is a vehicle for the transmission of the generative cell to the ovule. The generative cell consists mainly of nuclear material and while in the tube (rarely in the grain) it divides into two sperm cells, one of which will fertilize the ovule. In artificial media it is sometimes noted that, due to some deficiency in the medium, this division of the generative cell is irregular. In some cases,[24] after division has been initiated, a reversal of the process sets in and a restitution nucleus is formed.

IV. Experimental

A. *The Medium.* Basing themselves on the experience of other investigators (Brink,[13] Trankowsky[3] and Kuhn[14]) the authors found that a semi-solid medium of an agar or gelatin base was most suitable and shredded agar was to be preferred. Not all the pollen sprouted equally well in a given concentration of sucrose and agar, consequently the concentration was varied to obtain optimum growth. The sucrose concentration was between 5 and 10 per cent and the agar between 0.5 and 1 per cent. A small amount of cell-free fraction of boiled yeast extract was added in physiological doses (2 drops in 25 cc. *Cf.* Brink[13]).

B. *Mounting of the Medium.* In some cases petri dishes covered with a thin layer of agar were employed because they offered a large culture surface for comparative studies. On other occasions the media was mounted on slides, since this method is particularly well adapted for staining. Most often the medium was placed on cover slips.

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[24] Wulff, H. D., and Raghavan, T. S., *Planta*, 27 (1937): 466.

The cover slips or substitutes were cleaned in cleaning solution and sterilized in the ordinary manner. In addition they were irradiated with ultraviolet rays. Thus any influence which might conceivably be produced by foreign materials or growth was prevented.

The medium was applied to warm cover slips by means of a special glass applicator. It carried just enough material so that it could be smeared into a thin even layer.*

C. *Application of Pollen to Medium.* Two methods were employed in applying the pollen to the culture medium. In order to get an evenly-distributed, sparse culture, a camel hair brush was dipped into the pollen supply, which was struck against the side of the bottle to shake off the larger clumps of pollen, and was then held directly over the medium and tapped once or twice, strewing the grains on the substratum. Thus a fairly even distribution of the grains was insured. In the other method the point of the needle was applied to the pollen supply and the pollen adhering to it was placed in the slightly warm medium. The surface tension of the grains and the associated oil caused the small clusters of grains to spread.

D. *Culture Conditions.* Immediately after seeding, the cover-slips were placed in a moist chamber and were cultured in the dark at room temperature. For observation each cover-slip with the mounted culture was inverted and placed on a hollow ground tissue culture slide. Lanolin served as a seal to conserve the moisture within the chamber during the period of observation.

The relative position of the cover-slip had no effect upon the direction in which the tubes grew, as was verified by experiment. Apparently pollen tubes do not manifest polarity of growth nor gravitational response.

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*It was noted that the amount of medium employed in a given culture is a factor which influences the degree of germination.

E. *Application of the Growth-Promoting Substance to the Culture.* The material to be tested was embodied in the medium. Sugar agar was kept in stock at double the ordinary concentration. This was mixed 1 : 1 with an aqueous solution of the growth-promoting substance. The growth-promoting substances were only slightly soluble in water, nevertheless, the slightest traces in aqueous solution are very effective so that no difficulty was experienced in embodying physiological doses in the medium.

F. *Application of Vital Stains.* The same technique as described above was used in applying vital stains in low concentrations to the medium. The generative cells of the pollen tubes growing on such media absorbed the stain and became visible. Especially was this the case with the pollen of the *Amaryllidaceae.* With the generative cells thus visible, a great many interesting phenomena were observed regarding the position of the generative cell in the grain; the time of emergence from the grain into the tube; its movement in the tube plasm; the effect of cyclosis of the tube plasm on its movement; its behavior in the bursting tube; and its behavior after being ejected from the ruptured tube.

For these studies aqueous solutions of the following stains in the indicated concentrations were employed:

Neutral Red . . . . . 1 : 10,000 and 1 : 100,000;
Methylene Blue . . . . . 3 : 100,000;
Rhodamin B . . . . . 2 : 100,000;
Janus Green . . . . . 1 : 100,000;

Neutral Red was found to be the best vital stain for differentiating the generative cell and its vacuome from the tube plasm. Methylene Blue also proved to be satisfactory but did not differentiate these parts as well. In neither case did the tube plasm stain. Rhodamin B was not entirely satisfactory

but produced interesting effects. It stained the tube plasm as well as the generative cell, the latter however stained more intensely than the former, so that the two were still clearly differentiated. The growth of the tube was evidently disturbed when mere traces of this stain were added to the medium so that the tubes usually twisted, curled, bifurcated and branched; sometimes two or three tubes grew from a single grain. Apparently this substance affects the production of wall material. Janus Green proved to be a favorable stain for coloring the mitochondria in the tube plasm, but failed to stain the generative cell.

In most cases germination and the early growth of pollen tubes is not affected by the presence of vital stains in the nutrient medium. However, in general the tubes of vitally stained grains did not grow to the same length as the controls. Neutral Red is the least injurious of the stains studied. Very little difference in tube length was noted when this stain was used in low concentration. The study of the effect of these stains on the nuclear development of the generative cell will be taken up later.

G. *General Growth Phenomena.* Shortly after the pollen is sown on the agar, it imbibes water and increases in volume. This imbibition of water is a factor in the sprouting of the grains. The influx of water produces a swelling pressure in the plasm. The pressure from within causes the covering to break at the pores so that the tube emerges at one or the other pole. Usually only one tube develops; but sometimes two, rarely three grow. Older pollen fails to develop normally, the gametophyte bursting before a definite tube has been formed.

Sprouting is a matter of minutes when there is sufficient water in the medium. Pollen sown on relatively dry media (media spread in the usual way but allowed to dry in the air) failed to sprout until either the medium or the grain had absorbed water from the atmosphere of the moist chamber.

At first the velocity of the tube growth was slight but it reached a maximum in a relatively short time (20 to 30 minutes). After passing the maximum there was a steady diminution of the speed of growth. The most favorable growth periods of the tube varied with the kind of pollen employed. Eight hours is approximately the limit of favorable growth for liliaceous pollens. For other pollens the limit is considerably less, depending somewhat on the normal length of the tube.

Cyclosis is observed as soon as the tip of the tube is noticeable. During the growth of the tube, the plasm is constantly streaming to and from the tip in several channels. The rate of cyclosis is variable and not the same in different regions at any given time. From 80 to 100 microns per minute is a fair estimate of the average rate of cyclosis in normal tubes. In the immediate region of the tip the streaming is slower than the average, and the granules move to and fro rather than in a definite direction. Sometimes the granules are caught up again by the current and then move back in the direction of the body of the pollen grain. The forward movement of the plasm seemed to be along the wall of the tube and the backward flow, in the channels lying toward the interior.

There is some evidence that the physical nature of the plasm is different in different parts of the tube. Following germination, the coarsely granular contents of the pollen grain pass into the tube as it elongates. At this stage the short tube is densely crowded with variously-sized particles of fatty material. As growth proceeds many of the smaller fat bodies coalesce and there is a tendency for the material to accumulate in the region of the tip. As the tube lengthens the grain covering and the rear portions of the tube are regularly evacuated by the plasm. These regions are characterized by the formation of large vacuoles. The region in which the active plasm lies is separated from the evacuated region by one or more plugs.

The granular material becomes less and less; it, undoubtedly being digested and utilized in the growth process, until it has disappeared. It is a significant fact that in tubes possessing little granular material no further elongation takes place. However, many tubes cease to elongate before their reserves are fully utilized. Growth-promoting substances, or their deficiency, are most probably the factors, rather than the lack of food supply, that intervene to check the growth in such cases.

The most frequent cause of premature cessation of growth is bursting. When a tube bursts at the tip, the elongation is abruptly halted, the contents oozing from the rupture. Frequently, before bursting there is a bulging at the tip and a cessation of forward motion. Obviously the balance which normally exists between the turgor and the growth at the tip is disturbed. Apparently the influx of water continues at the normal rate but growth at the tip fails. The weak wall is stretched into spherical shape at the tip by the turgor until it is strained beyond the limits of elasticity to the breaking point.

H. *Results.* Over a period of two years pollens of more than 125 species of monocotyledonous and 50 species of dicotyledonous plants were tested. The authors collected most of the pollen in the Irwin Krohn Conservatory. Other common types were found in the neighborhood of the laboratory.

In the table are listed the preferred types of pollen. In making the selections the authors had two characteristics in mind: the retention of viability for a comparatively long period of time; and the development of relatively large tubes. The object was to find the most suitable kind of pollen, which in culture would serve for testing the effectiveness of growth-promoting substances.

In the table, Column 2 gives the size of the pollen grain in microns. The figures represent an average of at least 10 read-

TABLE I

List of Suitable Pollens for Testing Growth-Promoting Substances

Kind of Pollen	Size of Grain in Microns	Diameter of Tube in Microns	Viability in Months	Characteristic Features
Lilium auratum	165—90	20	10	much oil (yellow) present
Lilium candidum	120—90	18	6	much oil (yellow) present
Lilium rubrum	130—80	18	4	much oil (red) present
Lilium tigrinum	120—70	..	3	much oil (brown) present
Hemerocallis flava	145—85	20	1	rapid growth of tubes
Hymenocallis tubiflora	190—110	21	5	generative cell pigmented
Amaryllis (variegated species)	110—85	18	6—8	generative cell visible, takes vital stain readily
Crinum asiaticum	70 (round)	12	5	tubes grow straight
Narcissus jonquilla	60—30	10	3	tubes manifest a tendency to branch

ings. These measurements were made on pollen which had been in the medium at least a quarter of an hour. The grains of dry pollen are longer and thinner than is indicated in the table. The values obtained for the diameters of the tubes (Column 3) were nearly constant for normal individuals of a given species, at different distances from the grain, throughout the period of development. The values given are averages of at least 20 readings.

The viability, which is recorded in Column 4, expresses the results obtained in many trials. The figures given are not to be considered absolute since they are dependent on many circumstances affecting viability. The methods of storage may affect the viability. For details regarding the different methods of storing pollen and different effects under various conditions the authors refer the reader to Holman and Brubaker,[25] and Pfeiffer.[26] It must furthermore be remembered that the viability for a given kind of pollen may vary from year to year.

All the pollens listed in the table were stored in the same way. The anthers were allowed to open fully on the flower, and were then removed and placed in a paper-covered petri dish, where they were dried for several days. The pollen was removed from the anthers with a brush and placed in a light-tight container and preserved in a refrigerator.

The characteristics that are listed in the last column, summarize some outstanding features helpful in choosing the pollen. They will be further discussed.

V. Discussion

A. *Liliaceous Pollen.* All of the liliaceous pollens were found to be well adapted to the purpose of the experiments. *Lilium auratum* is probably the best and the most reliable species for

[25]Holman, R. M., and Brubaker, F., *Univ. Calif. Pub. Botan.*, **13** (1926): 179.
[26]Pfeiffer, N. E., *Contr. Boyce Thompson Inst.*, **9** (1938): 199.

the proposed test of growth-promoting substances. The size of the tube, the vigorous growth and the long viability make it particularly practical. The pollen is produced in great abundance by the plant. A volatile oil, soluble in ether, alcohol, water, and weak alkali is associated with this and other liliaceous pollens (*L. candidum*, *L. rubrum*, and *L. tigrinum*). The oil gives the fresh pollen a glossy appearance and causes the grains to adhere together, so that clumps are formed. Old pollen is dull in appearance and powdery. In very old pollen the oil is no longer present. Pollen more than six months old is of this nature. Ether extracts of fresh pollen, and of old powdery, non-glossy pollen of *Lilium rubrum* (8 months old) were made, and the relative optical densities of the extracts were determined with a colorimeter. The densities expressed in arbitrary units based on ether were in the following proportion—ether : extract from fresh pollen : extract from old pollen : : 1 : 69 : 3. The old pollen had evidently lost practically all of its volatile oil.

The oil is yellow and is distributed over the surface of the pollen grain and is lodged in the meshes of the lattice markings on the exine. When pollen grains are observed under the microscope at the time of sowing them on the medium, it can be noted that the oil leaves the meshes and is distributed as droplets over the surface of the medium. As many as 400 droplets of oil from one fresh pollen grain were counted, ranging from 15 microns to mere fractions of microns in diameter.

When a mass of such pollen is sown, the grains adhere momentarily and then spread apart, so that a colony is formed, in which the grains are almost equidistant from each other. The oil droplets appear between the grains and for a short distance beyond the limits of the colony (400 microns).

Since these *Lilium* pollens contain an abundance of oil, which is of a growth-promoting nature, or is the vehicle of a

natural auxin, any growth-promoting substance that is added to the medium (as is done in a test) might make the final concentration greater than the optimal physiological dosage. In this case the final effect would be inhibitory instead of stimulating. If this difficulty actually exists, these pollens might prove to be less satisfactory than was at first expected. This phase of the investigation has not yet been completed.

B. *Hemerocallis Pollen.* The pollen of *Hemerocallis flava* is plentiful in season. It contains a goodly supply of yellow oil. Its wide variation in grain size and short period of viability are disadvantages, but the abundance of fresh material in season, and its rapid growth are favorable factors recommending it for testing growth-promoting substances. The tubes of this pollen burst more readily than most other kinds. Elsewhere the authors have shown that the bursting is probably due to a lack of the deposition of wall material at the tip of the tube, so that the wall at this point can no longer resist the rapidly increasing turgor of the tube. The speed of growth in the *Hemerocallis* is unusually great so that it is probable that the turgor increases too rapidly for the slowly growing tip. If the turgor is so great that the weak wall at the tip is stretched beyond the limits of elasticity, evidently the tube must burst. This indicates a correlation between the unusually great speed of growth and the frequency of bursting.

C. *Hymenocallis Pollen.* The pollen grains of *Hymenocallis tubiflora* are the largest of those listed. The large size of grain and tube, the slight amount of associated oil, and the high degree of viability even after long periods of storage make this pollen a favorable means for testing growth-promoting substances. Unfortunately, the pollen cannot be secured as readily as the others.

This pollen is particularly well adapted for testing growth-promoting substances of the bios group, because it posseses an unusual generative cell which is pigmented,[15] The natural

pigmentation of the nuclear material in this cell makes it possible to investigate the development and the behavior of the generative cell in the living gametophyte and the effect produced by added growth-promoting substances. The orange-colored generative cell can be seen through the grain covering and is easily studied in the tube. A vacuome which lies midway between the poles in the broadest region of the ovoid or elongated cell, separates the pigmented material almost completely. A more detailed description was given in an earlier work.[1]

The pistil of this species is very long (20 – 22 cm.). In the cultures the tubes never exceeded a centimeter in length. Evidently some material necessary for normal development was lacking, or some inhibitory factor was present. The pigmented generative cell did not complete its division within the culture tubes. It increased in length in the tube (80 to 140 microns) to such a point where division was expected but was not realized. Staining with aceto carmine showed that a restitution nucleus was formed. Apparently, this behavior is abnormal since in our liliaceous pollens and in others it was noted that the nuclear phases of division of the generative cell did not differ from ordinary mitosis. This is in agreement with the reports of other investigators for other species (Trankowsky,[3] Cooper,[27] Eigsti[28]).

It seems that the lack of growth-promoting substances of the auxin group and the bios group prevent the normal growth of the tube and the normal division in the generative cell. Efforts were made without success by Wulff and Raghavan[24] to improve the medium so that the cultured growth might approach more nearly the normal. Their improvements apparently did not include the addition of substances of a growth-promoting nature.

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[27]Cooper, D. C., *Botan. Gaz.*, **98** (1936): 169.

[28]Eigsti, O. J., *Proc. Oklahoma Acad. Sci.*, **19** (1939): 105.

Eigsti[28] was able to inhibit the normal division of the generative cell and cause the formation of a restitution nucleus by the addition of 1 per cent and 0.1 per cent aqueous solution of colchicine to the medium. This suggests that the stunting of the tube and the formation of a restitution nucleus might be caused by some inhibitory factor which is present in *Hymenocallis* pollen. It is of course possible that some substance present in the pistil, and lacking in the pollen, would prevent the effect of any possible inhibitor, or it might supply the necessary growth-promoting substance, which is lacking in the pollen.

D. *Amaryllis Pollen. Amaryllis* (variegated species) is an excellent pollen to use for growth-promoting tests. The oil is not prominent although the pollen will respond to typical colony growth. The generative cell is not pigmented but its optical differentiation is always sufficient to distinguish it in the tube, even through the grain covering. The form of the cell and its vacuome are much like that of *Hymenocallis tubiflora.*

The generative cell showed unusual ability to absorb vital stains from the medium. In the experiments, neutral red, methylene blue and rhodamin B gave positive reactions, but Janus green, negative. An attempt was also made to stain with an anthrocyanin compound, which was extracted from the basal petal cells of the Black Tulip, but without much success. Apparently the blue aqueous extract had an inhibitory effect on the development of the tube and the generative cell. This attempt was made because it was suspected that the pigmentation of the nucleus described by McWhorter[29] was an altogether different phenomenon than that observed by the authors in *Hymenocallis.*

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[29]McWhorter, F. P., *Science,* 88 (1938): 411.

In other series of experiments the authors succeeded in staining the nuclei of epidermal cells of the etiolated sunflower seedling with the extracted material from the tulip. The staining in these cells was not uniform, just as it is not uniform in the intact tulip-petal cells of McWhorter, but the degree of staining had about the same distribution in the epidermal cells of the seedlings as was found in the normal tulip petals. This leads to the conclusion that McWhorter's stained nucleus is the result of the naturally occurring vital stain which does not affect all nuclei equally well. The authors do not feel justified in correlating the staining power with age, even though the youngest cells did not show stained nuclei, neither in the tulip petals nor in the seedlings. What is of particular interest here, is, that the aqueous extract had an inhibitory effect on the growth of the pollen tube and the development of the generative cell.

The ability of the generative cell to absorb vital stains from the medium made this pollen ideal for the study of the generative cell, its movement, and the effect of cyclosis.

Complete division of the generative cell into two sperm cells was noted under ideal culture conditions while the pollen was fresh. However, the phenomenon was not general, and was never observed when the vital stain was used.

Crinum asiaticum apparently did not contain much oil, but, like the other *Amaryllis* pollen, did respond to colony growth. The growth of the tubes is comparatively straight. Here also in some few tubes the generative cell is optically distinguishable and will readily take up vital stains.

Narcissus jonquilla was of special interest because of the characteristic branching of the tubes.

VI. Summary and Conclusions

1. For two years the pollen of more than 125 species of monocotyledonous and 50 species of dicotyledonous plants

were cultured on artificial media, for the purpose of determining the most favorable medium, and discovering a satisfactory kind of pollen which would serve for testing the physiological effectiveness of growth-promoting substances.

2. A semi-solid medium of agar, containing 10 per cent sucrose and a trace of yeast extract was found to be the most favorable for optimum growth.

3. Growth-promoting substances and vital stains are most effective when they are incorporated in the medium. Neutral red and methylene blue are the most favorable stains.

4. Pollen from monocotyledons gives better results than pollen from dicotyledons. The pollen of liliaceous plants is best but that of Amarylidaceous is also good.

5. The grains of the pollen from liliaceous plants are large, remain viable for months, but carry considerable quantity of volatile oil, which is of a growth-promoting nature or serves as a vehicle for growth-promoting substances. The tubes, which develop quickly and vigorously, have a considerable diameter. This pollen is particularly well-suited for testing the physiological effectiveness of the auxin factors.

6. The pollen of *Hymenocallis* and that of *Amaryllis* are particularly favorable for the study of the development of the generative cell and hence are to be recommended for the study of the physiological effectiveness of the bios factors.

LITERATURE CITED

Amici, G. B., *Ann. Sci. Nat. Botan.*, I, 2 (1824): 41; I, 21 (1830): 329.

Beck, W. A., and Donnelly, Sr. M. W., *Studies Inst. Divi Thomae*, 2 (1939): 179.

Beck, W. A., and Joly, R. A., *Nature*, 145 (1940): 226; *Trans. Am. Microscop. Soc.*, 60 (1941): 149.

Brink, R. A., *Am. J. Botan.*, 11 (1924): 283, 351, 417.

Camerarius, R. J., De Sexu Plantarum, 1694.

Cooper, D. C., *Botan. Gaz.*, 98 (1936): 169.

Eigsti, O. J., *Proc. Oklahoma Acad. Sci.*, 19 (1939): 105.

Fitting, H., *Z. Botan.*, **1** (1909): 1; **2** (1910): 225.

Fritzsche, K. J., *Poggendorf's Ann. Physik Chemie.*, **32** (1844): 481.

Hofmeister, W., Die Entsehung des Embryo der Phanerogamen, Leipsig, 1849.

Holman, R. M., and Brubaker, F., *Univ. Calif. Pub. Botan.*, **13** (1926): 179.

Kuhn, E., *Planta*, **27** (1937): 305.

Lidfross, B., *Jahrb. wiss. Botan.*, **29** (1896): 1.

Loofbourow, J. R., and Dwyer, Sr. C. M., *Studies Inst. Divi Thomae*, **2** (1939): 155. Morgan, Sr. M. N., *ibid.*, **2** (1939): 137. Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M., *ibid.*, **1** (1937): 163.

McWhorter, F. P., *Science*, **88** (1938): 411.

Mangin, L., *Soc., Botan. France Bul.*, **33** (1886): 337, 512.

Mohl, H. von, Beitrage zur Anatomie und Physiologie, Berlin, 1834.

Molisch, H., *Sitzungsber. Wien Akad. wiss. Math. Naturw.*, **102** (1893): 423.

Pfeiffer, N. E., *Contr. Boyce Thompson Inst.*, **9** (1938): 199.

Schacht, H., *Ann. Sci. Nat. Botan.*, III, **15** (1851): 80. *Jahrb. wiss. Botan.*, **1** (1858): 193.

Schleiden, M. J., *Wiegman's Arch.*, **1** (1837): 289.

Strasburger, E., Neue Untersuchungen über den Befruchtungsvorgang bei den Phanerogamen als Grundlage für eine Theorie der Zeugung, Jena, 1884.

Thimann, K. V., and Went, F. W., Phytohormones, Macmillan Co., New York, 1937.

Tieghem, P. van, *Ann. Sci. Nat. Botan.*, V, **12** (1869): 312.

Tokugawa, Y., *J. Coll. Sci. Imp. Univ. Tokyo*, **35** (1914): 1.

Trankowsky, D. A., *Planta*, **12** (1931): 1.

Wodehouse, R. P., Pollen Grains, McGraw-Hill, New York, 1935.

Wulff, H. D., and Raghavan, T. S., *Planta*, **27** (1937): 466.

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INVESTIGATIONS OF TENSION FIELDS IN PLASMA CULTURES*

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Ever since the supposed detection of radiations emanating from living biological materials (termed by Gurwitch "mitogenetic rays"), the actual existence of these rays from tissues during mitosis has been questioned by some investigators and substantiated by others. The entire subject of mitogenetic phenomena is still viewed askance by many who work with biological materials. Attempts have been made repeatedly to detect these radiations from growing onion-root tips, yeast cells, sea-urchin eggs, tumor tissue, etc. Some investigators claim to have detected their presence, while others have obtained altogether negative results. The presence of these mitogenetic rays is said to be indicated indirectly through their ability to cause neighboring tissues within the sphere of influence to start dividing. To the knowledge of the authors, these mitogenetic rays have never been studied directly but only through the effect produced upon the division rate of other cells.

Recently a paper[1] was published from these laboratories dealing with the growth-promoting substances liberated by traumatized tissues *in vitro*. Although the interest of the authors at the time was mainly centered around the resultant growth of tissue after injury, they observed and made mention of a fairly consistent pattern of outgrowth from the cut ends of the embryonic chick intestine cultured in chicken plasma and Drew's solution. In many cases a fan-shaped outgrowth

*Research conducted under the Sir Charles F. Williams Grant.

[1]Fardon, J. C., Sullivan, W. A., and Andrus, Sr. M. B., These Studies, 2 (1939): 233.

proceeded from the two cut ends after from 24 to 40 hours of incubation. These fan-shaped outgrowths, consisted of a mixture of fibrocytes and epithelium.[2] After some 65 to 100 hours of incubation the growth pattern changed to a sort of single or double loop formation of narrow but rather densely packed cells.[3] In most instances the plasma within the loops was found to be liquefied with a few scattered cells afloat and with some adhering to the coverslip.

Just how much of this peculiar growth pattern is due to the liquefaction of the plasma clot is not yet known; however, during the early growth stages of the tissue explant the nature of the outgrowth to a certain extent indicates the potential pattern eventually formed. The nature of the outgrowth on the whole bears a rather striking resemblance to the field around a bar magnet. During the early stages of *in vitro* existence the growth zone may be likened to a bar magnet with a weak field; as the tissue becomes older and the growth more abundant, the appearance of the growth figure is then more like that obtained from a bar magnet with a strong magnetic field.

Some months later,[4] during the course of experiments *in vitro* with embryonic chicken heart, a similar phenomenon was observed, which, though presenting a somewhat different picture, nonetheless furnished further evidence of the existence of some force guiding the outgrowth of cells in a definite pattern between the two explants which the authors have designated as "tension fields." In one of these experiments two fragments of embryonic chicken heart were accidentally introduced into the plasma drop of a slide culture. When growth measurements were made a day later, the phenomenon

[2] *Loc. cit.*, Plate I, Fig. 3 and 5., p. 236.

[3] *Loc. cit.*, Plate II, Fig. 6 and 9, p. 236.

[4] Fardon, J. C., Brotzge, G., Loeffler, Sr. M. K., and Breit, J., *Nature*, **146** (1940): 619.

PLATE I

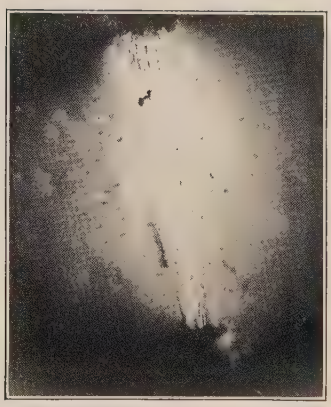


Fig. 1

Fig. 2

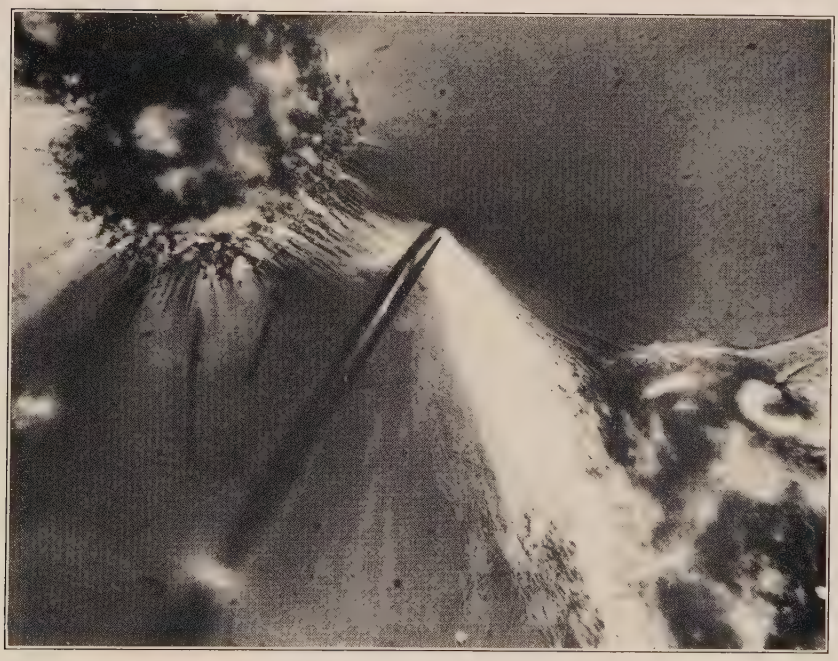


Fig. 3

of "tension fields" was observed in the culture containing the two pieces of embryonic heart. This field was found to exist between the two pieces of growing tissue and was made visible through what seemed to be slight differences in the refractive index of the plasma clot. The intensity of the field seemed to be greatest along a straight line joining the two pieces of tissue.

A related phenomenon of interest which tends to confirm the presence of actual tension between the tissue fragments is the growth zone or growth pattern within it. It is familiar to all who have worked with tissue culture that an isolated fragment of tissue usually exhibits a zone of new growth and migratory cells in a radial fashion, i.e., the cells appear to have originated at the periphery of the tissue explant and continue radially and with fairly uniform distribution into the plasma medium. However, where a tension field is in evidence between two fragments of tissue in the same plasma clot, the uniform radial growth is slightly but definitely altered. The outgrowth of cells from those portions of the tissue which lie closest together does not form a sector of radial growth, but rather a bundle of parallel elongated cells along a line joining the centers of the two pieces of tissue. When observing both fragments of tissue at the same time under low-power magnification, both the "strains" set up in the medium and the outgrowth of cells strongly indicate the presence of a force directed between the two pieces of tissue.

The authors' observations are confirmed by certain evidence in photographs accompanying an early experiment by Carrel.[5] While studying the effect of embryonic juice on the growth of connective tissue *in vitro*, Carrel made a series of photographs demonstrating his experimental results. In these experiments he explanted several tissue fragments in one culture flask and, after some 48 hours, during which extensive growth occurred,

[5]Carrel, A., *J. Exptl. Med.*, **18** (1913): 287.

his photographs revealed dense bridges of outgrowth connecting the tissue fragments. These multiple cultures (as far as can be judged from the photographs accompanying his article) were in every respect similar to those of the authors after the tension field was completely gapped by the growing and migrating fibrocytes. Carrel, however, made no mention of this phenomenon other than to remark that when two fragments were sufficiently close together they became bridged by cells. Neither did he mention the presence of a tension field prior to the formation of the cellular bridge.

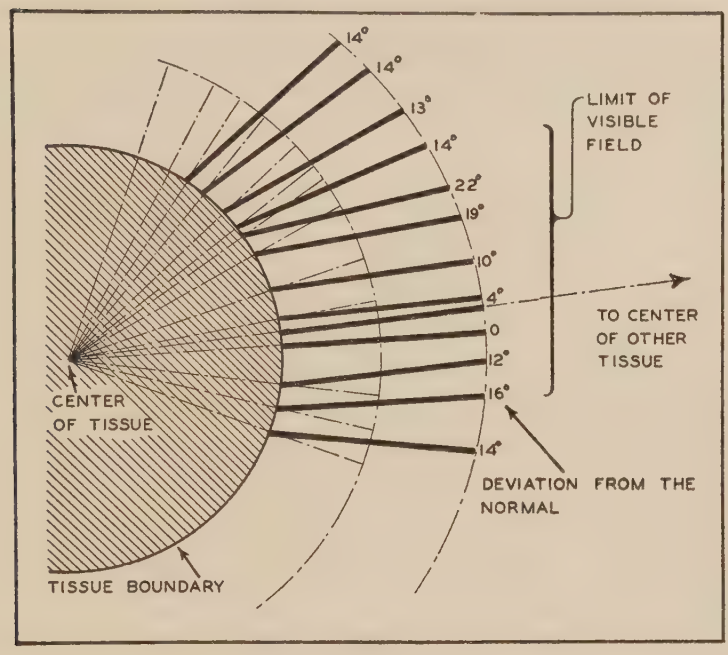
EXPERIMENTAL

In the preparation of the cultures standard procedure was followed. Two pieces of tissue were placed upon the coverslip and a drop of chicken plasma added. The tissue fragments were usually separated by a distance of 1 to 2 mm. The first observations were made about 18 hours after incubation and the second about 40 hours from the time of explanation. The cultures were usually discarded shortly before the onset of degeneration. No sub-cultures were made. A total of about 1,500 cultures were prepared and among these some 157 showed positive evidence of the attraction field.

Considerable care had to be exercised in the observation of the fields for many of the elongated cells which migrated from the explant appeared much like rays when viewed out of focus. It was necessary to focus continually upon various depth levels of the plasma clot between the two pieces of tissue to make certain that migratory cells out of focus did not contribute to the true picture of the "attraction field."

Fig. 1 is a photomicrograph of the original culture containing two fragments of 8-day embryonic chicken heart. In this culture the field is unequivocally demonstrated. In Fig. 2 a portion of the field and tissue fragment is shown under high-power magnification. It will be noted at once that the

FIG. 4—*Diagramatic Representation of the Deviation of Growth from the Normal*



outgrowth within the field differs from the picture ordinarily observed in a tissue culture containing only a single fragment. In the immediate vicinity of the field the outgrowth or migratory fibrocytes have aligned themselves in a parallel bundle. The deviation of the fibrocytes from the normal radial growth or migration within the field is represented diagrammatically in Fig. 4. The diagram was constructed by tracing the most clear-cut cells on the photograph and representing them schematically in the diagram shown in Fig. 2. The broken lines represent radii from the center of the tissue explant. The solid lines, originating from the boundary line of the tissue, indicate the direction of the growth of fibrocytes, singly or in small individual bundles. The angle of deviation from the

normal is taken from each focus of outgrowth and is given in degrees. It will be noted that the deviation is practically zero near the line drawn between the centers of the two fragments of tissue. The parallel bundle at the periphery of the tissue explant is included in an angle of about 46°, and the total angle in which deviation from the normal growth persists is about 77°.

In a few cultures three pieces of embryo heart tissue were placed in line with a distance of 1 to 1½ mm. between each piece. After 24 hours incubation, fields and fibrocytes were observed between all three fragments. It has been observed that in all instances continued growth bridges the tissue fragments with elongated fibrocytes oriented parallel to the field.

In order to determine if the fields detected could be due to non-living material, a large number of slides were prepared containing two grains of sterile sand in a plasma drop. In no case were tension fields observed after an extended period of incubation.

An experiment was conducted to determine if both fragments of tissue must be living in order that the field be manifested. A piece of embryonic heart was placed in Drew's solution and subjected to a temperature of 100° C. for 15 minutes. This tissue was then cut into small pieces and cultured with living embryonic heart tissue. About 48 cultures were prepared and in no instance was a field observed.

To determine whether a tension field could be obtained with tissue other than embryonic, 48 cultures were prepared using cardiac tissue from a week-old chick. Although some cells migrated from the explants, no fields were observed.

In order to establish any possible effect of embryo juice on the formation of tension fields, an experiment was conducted in which plasma and embryo juice were used in equal proportions in cultures of 8-day chick embryo heart. No fields

were observed. However, since only 24 cultures were prepared for this experiment, one cannot be certain that the presence of embryo juice prevents the appearance of the field.

To determine whether tension fields could be obtained between tissues taken from different species of animals, 96 cultures were prepared in which a piece of embryonic chicken heart was placed near a piece of mouse embryo heart. Chicken plasma and Drew's solution were used for the media. In only two of the cultures could scant evidence of the field be detected. These fields were so weak, however, that no attempt was made to photograph them.

Recent investigations have shown that the tension fields become more prominent when observed under polarized light. These observations again lead to the conclusion that some force must exist between pieces of living tissue which is capable of setting up strains in the plasma membrane.

Cultures of 8-day chick embryo heart tissue, permitted to remain at a temperature of 18°-20° C. for a period of 24 hours, gave no evidence of tension fields, nor exhibited growth or migration, though they remained alive, as indicated by continued beating. Such cultures, when transferred to an incubator at 37.5° C., showed growth and migration of fibrocytes after 12 to 24 hours with the appearance of a considerable number of tension fields. The experiment thus proved the tension fields not to be the result of a physical strain produced by the mere presence of the tissue fragments, but to be concomitant with growth and migration under optimum physiological conditions.

Exploration of the area between the two tissue fragments with the micromanipulator showed that the field was made evident by a very thin membrane under considerable tension. The membrane was found to be pleated between the two tissue fragments and this pleating was observed to be the factor

which rendered the "tension field" visible. A photomicrograph taken with polarized light, (Fig. 3), shows the pleated membrane displaced by the micromanipulator needle. Further exploratory investigations throughout the plasma drop showed that the membrane was of the same diameter as the plasma drop, but separated the latter into an upper and lower stratum. Such membranes were also found to exist in plasma drops without tissue fragments, but lacking, of course, the visible tension fields.

Whether the tension fields observed by the authors bear any relation to the plasma fibrin strands in plasma membranes produced by Doljanski and Roulet[6] is not yet known. The experimental procedure employed by these investigators is quite different from the authors'. The former applied tension to plasma membranes stretched over glass frames and found cellular growth of explanted tissue oriented in line with the fibrin strands.

CONCLUSIONS

Membrane pleating produced by "tension fields" can be observed between pieces of living embryonic tissue through what seems to be a difference in the refractive index of the plasma clot. It has been shown conclusively, though for embryonic tissue alone, that the tension fields do not appear unless the tissues are cultured under optimum physiological conditions.

Since tension fields have not been observed between one living and one dead piece of tissue, and since they do not appear in cultures which have been kept at a lower temperature than is normal for tissue cultures, it seems quite evident that growth or migration is a prerequisite to the formation of the fields. This is further substantiated by the fact that adult

[6]Doljanski, L., and Roulet, Fr., *Arch. Entwicklungsmech. Organ.*, **131 (1934)**: 512.

tissue, which will not grow in plasma alone, does not give rise to such fields. From the investigations conducted so far it is evident that three problems should be investigated: (1) the cause of the migration of cells from one piece of tissue toward another piece of living tissue; (2) the cause and nature of the membrane in the plasma clot; and (3) the characteristics of the field existing between the pieces of living tissue and factors which can influence this field.

SUMMARY

1. Out of a total of 1,500 cultures, 157 gave positive evidence of "tension fields."

2. The field was observed in cultures of chick embryo heart, intestine, and skin.

3. The field is not visible in cultures containing one living and one dead piece of tissue.

4. Adult tissue did not show these fields.

5. No fields were observed in cultures containing embryo juice, though this is not conclusive because the number of experiments was insufficient.

6. The tension fields are rendered visible by a pleating of the plasma membrane within the clot.

7. Elongated fibrocytes invariably follow the parallel streamers within the field.

8. Plasma membranes also have been observed in cultures not containing explanted tissue.

BIBLIOGRAPHY

Carrel, A., *J. Exptl. Med.*, **18** (1913): 287.

Doljanski, L., and Roulet, Fr., *Arch. Entwicklungsmech. Organ.*, **131** (1934): 512.

Fardon, J. C., Brotzge, G., Loeffler, Sr. M. K., and Breit, J., *Nature*, **146** (1940): 619.

Fardon, J. C., Sullivan, W. A., and Andrus, Sr. M. B., THESE STUDIES, **2** (1939): 283.

SOLUTE PRODUCTION IN GROWING CORTICAL CELLS OF HELIANTHUS ANNUUS SEEDLINGS*

By WILLIAM A. BECK, ALFRED LONSING, and SISTER M. BASILIA ANDRUS, O.P.

Introduction

This study is a contribution to the determination of the growth characteristics of Helianthus seedlings. The influence of auxin as a growth promoter is well established, but the manner in which it causes cell enlargement is obscure. Active growth of the wall by intussusception during cell enlargement can hardly be doubted.[1] The question arises: Is it sufficient by itself to cause the enlargement? If the growth of the wall is the only manifestation of the energy involved, then the necessary suction tension required for the influx of water in the cell sap cavity would be due entirely to the reduction of the wall pressure. In such a case the normal osmotic pressure of the cell sap would necessarily decline and it would be inversely proportional to the growth of the cell. If, however, the production of solute is involved, the total solute present at any time should show some relation to the growth. Beck[2] showed that for the epidermal cells of comparable plants, the production of solute is proportional to the growth. The dual purpose of the present work is to determine the relative amount of solute which is produced in the growing cells of the cortical parenchyma, and to compare the amounts of solute produced in the cortical cells with that produced in the epidermal cells, so that some conclusions might be drawn from the data

*From the Bradley Hall Laboratories of the Institutum Divi Thomae, Palm Beach, Florida.

[1]Söding, H., *Jahrb. wiss. Botan.*, 79 (1933): 231.

[2]Beck, W. A., *Plant Physiol.* (In press). Beck, W. A., and Donnelly, Sr. M. W., These Studies, 2 (1939): 259.

regarding the possible participation of turgor in the process of growth.

Methods

The authors used etiolated Helianthus seedlings, grown under conditions identical with those previously used by Beck,[2] so that the results obtained might be comparable not only among themselves but also with those obtained by him for the epidermal tissues. The investigations were made in the same zones of the hypocotyl which Beck proved to be particularly favorable and which permitted a sufficiently comprehensive study of conditions in both the young and the mature cells. These zones were each 5 mm. long and extended from the base of the cotyledons to a point 35 mm. beyond. Sectioning was made under paraffin oil. The sections of cortical tissue were made as close as possible to the epidermis, but the epidermis itself was first cut away.

The length and the diameter of the cells were measured with the aid of the eye-piece micrometer. The determinations were made on sections which were cut and mounted in paraffin oil. Under these conditions the tissue section did not imbibe or lose water; but water may conceivably have passed from one cell to another within the same section. Any error due to such movement of the water from cell to cell was probably negligible and could not seriously affect the average reading and consequently no effort was made to determine the possible error. In some cases the sizes of the cortical cells were determined while the epidermis remained attached to the sections and the results did not differ appreciably from those obtained when the epidermis was removed. The accepted values are the averages of not less than 30 independent determinations made in the uppermost, the mid, and the lowermost regions of each zone. (*Cf.* Table 1, Column 3).

TABLE I

The Size of Growing Cortical Cells of Helianthus Annuus Seedlings and the Amount of Solute Which They Contain

Zone	Distance in mm. from Base of Cotyledon.	Length of Cells in Microns.	Average Length of Cells in Microns.	Average Equivalent Concentration of Cell Sap in Mol. ($C_{12}H_{22}O_{11}$)	Total Solute Present; Microns × Mol.	Average Volume of Cells; Microns^3.	Total Solute; Microns^3 × Mol.
1	0.83	41.50	48.34	0.405	19.58	80336	32536
	2.50	47.70					
	4.17	55.83					
2	5.83	66.35	80.67	0.398	32.11	145980	58100
	7.50	81.27					
	9.17	94.40					
3	10.83	106.22	116.65	0.384	44.79	202494	77757
	12.50	116.46					
	14.17	126.28					
4	15.83	135.68	144.51	0.344	49.71	261505	89957
	17.50	144.66					
	19.17	153.21					
5	20.83	161.34	168.90	0.339	57.26	305641	103612
	22.50	169.04					
	24.17	176.32					
6	25.83	183.25	189.90	0.309	58.68	343317	106085
	27.50	189.82					
	29.17	196.11					
7	30.83	202.12	207.77	0.294	61.08	375980	110538
	32.50	207.84					
	34.17	213.35					

The normal concentration of the sap was calculated from the osmotic value of the tissue at incipient plasmolysis, the volume of the cell in the same state and the volume in the normal state according to the equation of Ursprung:

$$O_n = O_g \times \frac{V_g}{V_n} \qquad (1)$$

in which O_n is the concentration of the cell sap in the normal state; O_g, the concentration at incipient plasmolysis; V_n, the volume of the cell in the normal state; and V_g, the volume at incipient plasmolysis.[3] The total solute present in a cell varies directly as the concentration of its cell sap and directly as its volume; therefore a satisfactory expression in arbitrary units can be obtained by the product of the normal volume of the cell and the normal concentration of the cell sap.

The authors' experiments, as well as those of previous investigators,[4, 5] showed that growing cells in etiolated plants, such as were employed in the present work do not increase their diameters appreciably but only their lengths. When making a comparison of the amounts of solute present in maturing cells of the same tissues it is consequently sufficient to express the values in mols $\times$ microns, (concentration of the cell sap multiplied by length of the cell) since the diameters of the cells remain practically constant. We expressed the total solute present not only in this manner but also in mols $\times$ microns^3 (concentration of the sap multiplied by volume of the cell). The latter expression was used in comparing the solute production of the cortical tissue with the solute production in the epidermal tissues. A comparison of the solute

[3] Ursprung, A., Handbuch der biologischen Arbeitsmethoden, Abt. XI, Teil 4, Urban and Schwartzenberg, Berlin, 1937. *Cf.* p. 1111.

[4] Ursprung, A., Handwörterbuch der Naturwissenschaften, Band 7, Fischer, Jena, 1932.

[5] Borgström, G., Monograph, William and Norgate Ltd., London, 1939.

produced in the two tissues when expressed in mols × microns is not satisfactory because the diameter of the cortical cells is considerably greater than that of the epidermal cells.

Results Obtained

In Column 4 of Table 1 the average length of the cortical cells is recorded. These averages were calculated from the actual figures obtained in the respective regions of each zone as recorded in Column 3. The average diameter of the cells of the first zone of the cortical tissue was 45.9 microns while in the six older zones it varied but slightly from 48 microns.*

The volumes of the cells were also calculated and are given in Column 7, Table 1. They were expressed in order to facilitate the comparison of the solute produced in the cortical and epidermal tissues respectively. In Column 5 an average value of the equivalent concentration of the cell sap is given in mols of sugar solution. The average value for the zone only is given since with the method employed it was not possible to obtain reliable results with tissue strips which were considerably shorter than 5 mm.

Discussion

A significant fact given in the data is that the cells enlarge by increasing their length, i.e., the lateral walls grow while the transverse walls fail to grow. This may be explained by the theory that growth occurs in the direction of the translocation of the auxin.[6,7,8] It is well established that auxin is trans-

*The average diameter of the epidermal cells in Zone 1 was 16 microns, and 18.5 microns in the cells of the six lower zones.[2]

[6]Ruge, U. von, *Z. Botan.*, 31 (1937): 1.

[7]Went, F. W., and Thimann, K. V., Phytohormones, Monograph, Macmillan Co., New York, 1937.

[8]Crafts, A. S., *Botan. Rev.*, 5 (1939): 471.

located through the phloem,[8] and the auxin which enters other tissues moves predominantly in the polar direction. Basipetal polarity of auxin movement has been observed by several investigators, for example, Went and Thimann,[7] and Crafts.[8] External factors can influence the phloem permeability and according to Borgström[5] may induce transverse streams of auxin in plants. Since no such agents induced transverse streams under the present experimental conditions there was auxin movement only in the basipetal direction and the growth of the cells was consequently polar.

The diameter of the cortical cells did not vary appreciably from 48 microns except in the first zone where the average diameter of the youngest cells was 45.9 microns. In strictly comparable plants Beck[2] found little variation in the diameter of the epidermal cells; the older cells were 18.5 microns in diameter and the youngest in the first zone 16 microns. The difference between the diameter of the epidermal and cortical cells of any zone remains the same; the diameter being almost three times as great in the cortical cells as it is in the epidermal. Other things remaining the same, this factor gives the cortical parenchyma a greater capacity for cell sap.

The average length of the cells in the first zone of the cortical tissue was relatively longer than the average length which Beck found in the epidermal cells, (45 microns average length of cortical cells as compared to 27 microns average length of epidermal cells). But in the lower zones the average length of the cortical cells was slightly shorter than that reported for the same zone of the epidermis, (207 microns average length of the cortical cells as compared to 221 microns the average length of the epidermal cells in the seventh zone[2]). The cells of the cortical tissue increased 4.3 times from the first to the seventh zone while the epidermal cells increased their lengths 8.4 times in the same regions. This is in general agreement

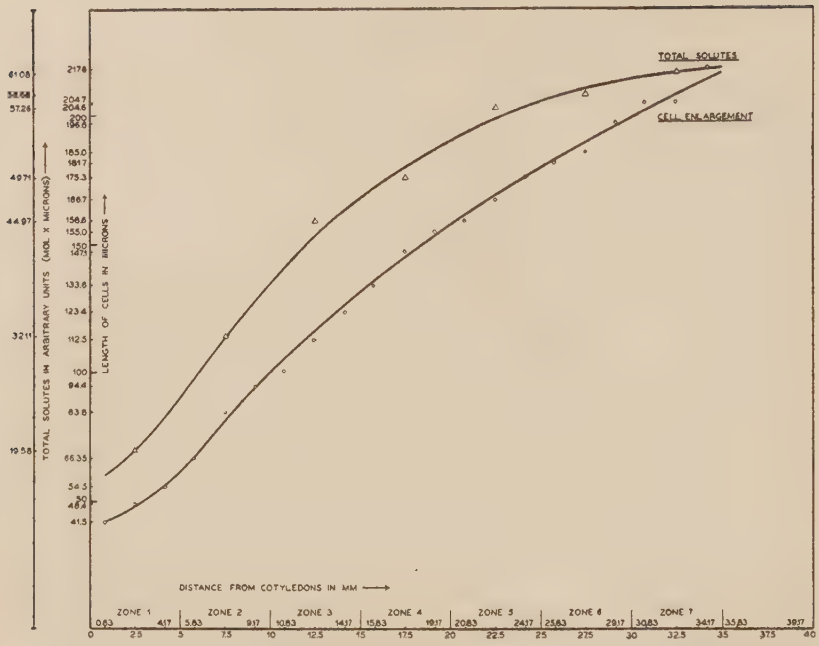
with the results recorded by Beck, Schocken, and Donnelly[9] for somewhat different regions of similar plants. Their investigations were made at 0–5 mm. and 40–45 mm. from the base of the cotyledons. In this connection it must be remembered that in determining the average size of the epidermal cells the region of the stomatal complex was avoided because the cells in this region were very irregular and did not permit a satisfactory comparison.

Two other explanations of this apparent difference in size of cells and rate of enlargement may be offered. First the cells of the lower zones of the cortical tissue may divide. This is contrary to the generally accepted opinion. However, we have occasionally found pairs of short cortical cells standing in tandem, the sum of whose lengths approximated the average length of the normal cells of the proper zone which would seem to indicate that mature cells divided. A second possible explanation is that the normal cortical tissue of the intact plant is under stress, so that the cells contract when tension is removed by sectioning. This would cause the length obtained experimentally to be shorter than the true length. If this is the correct explanation our results would indicate that tissue tension increases in the older zones.

The final value of the amount of solute present which was accepted and recorded in Table I, is not affected by the fact that the length obtained experimentally may not be the true length, since the product of the true normal size of the cell and the relatively lower (calculated) concentration of the sap must be the same as that which we obtained by multiplying the accepted size by the relatively higher concentration.

The average volume of the cortical cells of the first zone was about 16 times the average volume of the epidermal cells

[9]Beck, W. A., Schocken, K., and Donnelly, Sr. M. W., These Studies, 2 (1938): 107.

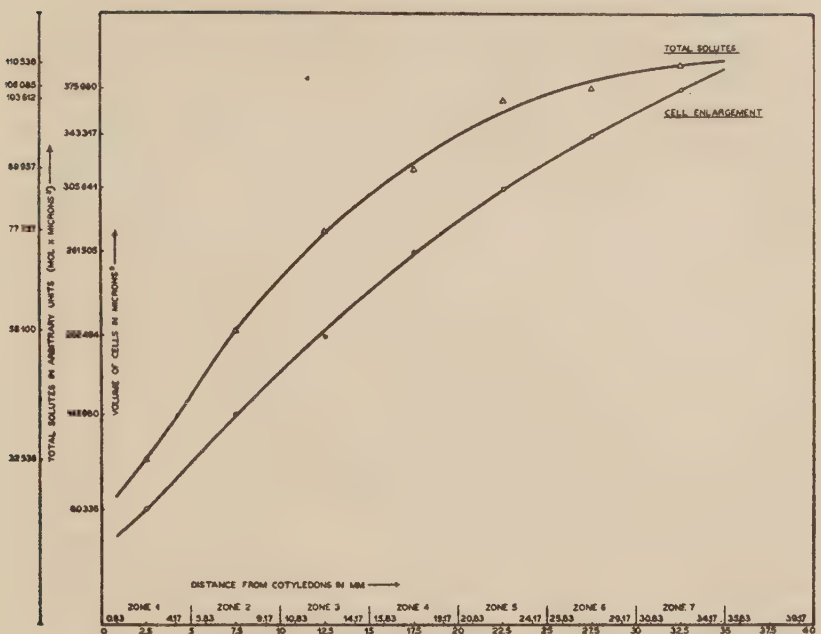


CELL ENLARGEMENT IN THE CORTICAL TISSUE OF THE HELIANTHUS HYPOCOTYLS AND TOTAL SOLUTES OF THE CELL SAP CAVITY EXPRESSED IN MICRONS × MOL

Fig. 1

of the same zones, but in the succeeding zones, the volume of the cortical cells was never much greater than 6 times that of the epidermal cells. It is remarkable that in the first zone the capacity of the cortical cells is so much greater than that of the epidermal cells. If it be assumed that the embryonal cells of the dynamic cortical tissue must be provided initially with a considerable quantity of reserve nutrient materials in order to be able to enlarge them, then the reason for the relatively great size of the cells of this tissue in the first zone becomes clear.

From the results it is evident that in the cortical parenchyma cells, as well as in the epidermal cells, solute is constantly produced during the process of growth. The curves (Fig. 1



CELL ENLARGEMENT IN THE CORTICAL TISSUE OF THE HELIANTHUS HYPOCOTYLS AND TOTAL SOLUTES OF THE CELL SAP CAVITY EXPRESSED IN MICRONS^3 × MOL

Fig. 2

and 2) in which the sizes of the cells and the amount of solute present are plotted against the distance of the cells from the base of the cotyledons (which may serve as an indicator of the relative age of the cells) are typical growth curves. The points of inflection are at a distance of 6.5 mm. from the base of the cotyledons. This is the same as in Beck's equivalent curves for the epidermis.[2] This shows that in the cortical tissue, as in the epidermal tissue, maximum growth occurs at this point. This is in agreement with the results obtained by Beck and Donnelly[10] who employed other methods.

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[10]Beck, W. A., and Donnelly, Sr. M. W., THESE STUDIES, 2 (1939): 179.

It made no significant difference whether the total amount of solute present was expressed as the volume of the cell multiplied by the molar concentration of the cell sap or as the length of the cell multiplied by the molar concentration. Both methods clearly showed that solute was constantly produced during the process of growth and that the production was approximately proportional to the growth of the cells. (*Cf.* Fig. 1 and 2).

It might be expected that the turgor of the cells under these conditions would be maximum at the point where growth is maximum. In studies, the results of which will be published later, the authors proved, however, that the turgor and the equivalent wall pressure is minimum at this point. This is in agreement with the findings of Beck[2] for the epidermal tissue of strictly comparable plants. It is also in agreement with the findings of Ursprung and Blum[11] for the growing zone of the root of *Vicia faba* seedlings. It is not difficult to explain the apparent paradox at this time, since it is now known that the walls of growing cells grow actively and cause the wall pressure and the equivalent turgor to decrease. This explanation is in agreement with the results of Söding[1] who showed that the first phase of growth is irreversible and that the stretching of the wall by turgor must follow this phase.

The amount of solute produced in the cortical tissue was found to be much greater than that produced in the respective regions of the epidermis. In order to compare these amounts and the factors involved a table was arranged. The ratios of the amount of solute present are particularly interesting. In the first zone the production of solute must be comparatively very great in the cortical tissue (*Cf.* Table II Column 4, Zone 1). In the second and third zones the production is still comparatively great, but in the remaining four, the cortical produces

[11]Ursprung, A., and Blum, G., *Jahrb. wiss. Botan.* **63** (1924): 1.

TABLE II

The Comparison of Total Solute Produced and Concentration of Cell Sap in the Epidermis and Cortical Parenchyma of Helianthus Seedlings

Zone	Total Solute in Microns^3 × Mols		Ratio Cortical : Epidermis	
	Epidermis	Cortical	Total Solute	Sap Concentration
1	1444.0	32536.0	1 : 22.5	1 : 1.50
2	5143.7	58100.0	1 : 11.3	1 : 1.60
3	7854.2	77757.0	1 : 10.1	1 : 1.54
4	10037.3	89957.0	1 : 8.9	1 : 1.50
5	10679.5	103612.0	1 : 9.7	1 : 1.50
6	12227.1	106085.0	1 : 8.5	1 : 1.37
7	12745.7	110538.0	1 : 8.6	1 : 1.30

on an average 8.5 times as much solute as the epidermis. In spite of the fact that much more solute is produced in the cortical tissue, the normal concentration of its sap is almost constantly only one and one-half times as concentrated as in the epidermis. The variation of the concentration in the cortical tissue was 0.405 mol in the first zone to 0.294 mol in the seventh. The concentration varied somewhat less in the epidermis, being 0.270 mol in the first zone and 0.226 mol in the seventh. Other things remaining the same, the greater production of solute in the cortical tissue and the constantly higher normal concentration of the cell sap would tend to make the suction tension of the cortical tissue greater than that of the epidermal tissue. Ursprung[4] and others pointed out that lower suction tension should be expected in the epidermis, since one of the functions of the epidermis is to serve as a water reservoir for the more internally located

tissues. Unpublished results for the suction tension of the epidermal cells and the cortical cells of our seedlings showed that the epidermal tissue actually does have a lower suction tension than the cortical tissue. The difference was not, however, as great as might have been expected from the ratios of the total solute present (*Cf.* Table II, Column 4), and the ratios of the normal concentration of the sap (*Cf.* Table II, Column 5). This is due to the fact that the epidermis produces a physical pressure which tends to reduce the suction tension of the cortical tissue.

These notions make it understandable why the cortical tissue should produce so much more solute than the epidermis since it is a particularly dynamic tissue and must maintain a relatively high suction tension in spite of the greatly increased capacity of the growing cells and the counter pressure of the epidermis which tend to reduce its suction tension.

There is an evident decline in the ratio of the solute present in the epidermal and cortical cells respectively in the last four zones. (*Cf.* Table II, Column 4). This would indicate that the rate of solute production which is very much greater in the cortical tissue than in the epidermal tissue declines sharply in the fourth zone. It is interesting to note that the decline occurs simultaneously with the probable changes in the cell wall in this same zone.

There were other indications that important changes occur in the cells in this region which forced the authors frequently to check their methods and individual determinations for this zone. They are not prepared at this time to say what these changes in the cells are, but they strongly suspect that the walls become less plastic and more elastic. This is in agreement with the results of Söding,[1] previously referred to in this work, and gives further evidence that the first phase of growth is irreversible, while the wall is still plastic and that older cells have somewhat elastic walls.

If the cell wall is enlarged by the active growth of the wall, then the wall pressure and the turgor tend to become less since less reaction is offered to the force of turgor. There would also be a tendency for the suction tension to be increased momentarily since the suction tension is given by the osmotic pressure of the cell sap less the wall pressure. From the formula of Ursprung[3]:

$$Sz_n = Si_n - W_n$$

where Sz_n symbolizes the suction tension of the cell; Si_n, the suction tension of the contents of the cell; and W_n, the normal wall pressure, it can readily be seen that with a decrease in W_n the value of Sz_n would momentarily become greater. The osmotic pressure of the cell sap would, in time, become considerably reduced because the capacity of the cavity was increased, so that eventually the turgor and the suction tension would both become very low and the cell could not long remain turgescent unless solute is constantly produced during the process of growth.

Our results give evidence that the rate of solute production is approximately proportional to the rate of growth. The consequence of the active growth of the wall and the simultaneous production of solute in the cavity is that solute production tends to maintain a turgor and suction tension, which serve the needs of the growing cell. As the cell wall becomes more rigid the wall pressure (W_n) and the turgor increase considerably, and the rate of solute production, and with it the suction tension of the contents of the cell (Si_n), decline. In consequence of both of these factors the suction tension (Sz_n) of the more mature cells must be less than the suction tension of relatively younger cells, according to equation (2). This conclusion is in agreement with the authors' direct determinations and with Ursprung's suction tension gradient.[4]

From the results it is evident that solute is constantly produced in the growing cells. It is reasonable to hold that the solute is elaborated by the protoplasm, probably as a physiological response to the stimulation of auxin. Assuming this to be the case it follows that growth by cell enlargement is more than an active growth of the wall. Both phenomena, the growth of the wall and the production of solute are equally important phases of growth. Growth by cell enlargement should consequently be regarded as a truly physiological process like the growth of the mass of protoplasm and growth by cell proliferation.

Summary and Conclusions

1. A study was made to determine the production of solute in the cortical parenchyma of etiolated sunflower seedlings and a comparison with the production in the epidermal tissue was made.

2. The determinations were made for the seven youngest zones of the hypocotyl, each zone being 5 mm. long.

3. The results were expressed in arbitrary units, microns $\times$mols and microns$^3 \times$mols, obtained by multiplying the length and the volume of the cells respectively by the normal concentration of the cell sap.

4. The dimensions of the cells were determined directly with the aid of the eye-piece micrometer. The average of 30 independent determinations were accepted as final.

5. The normal concentration of the cell sap was determined according to the plasmolytic method of Ursprung.

6. The cells did not increase their diameter appreciably, but greatly increased their length. This is in agreement with the theory that auxin is translocated in a basipetal polar manner.

7. The youngest cortical cells are much longer than the epidermal cells of the same age, which may be considered

evidence that they contain material which is necessary for the large quantity of solute that is required by this tissue, which has a greater capacity and a more dynamic function than has the epidermis.

8. As in the epidermis, solute is constantly produced in growing cells at a rate which is approximately proportional to the rate of growth. Maximum solute production and growth occur at a point about 6.5 mm. from the base of the cotyledons in both the epidermis and the cortical tissue.

9. The production of solute insured the necessary suction tension and turgor in the growing cells, thus preventing their collapse.

10. Considerably more solute is produced in the cortical tissue which is more dynamic than in the epidermal tissue which serves as a water reservoir. This is in harmony with the respective functions of the tissues.

11. The results indicate that growth by cell enlargement cannot justly be regarded as purely an active growth of the wall by a process of intussusception, but that solute is produced simultaneously with the growth of the wall. It is reasonable to assume that the material necessary for the increased mass of the wall and the solute which is stored in the cell sap are elaborated by the protoplasm.

12. It is probable that auxin acts upon the protoplasm and induces growth.

13. Growth by cell enlargement like other forms of growth should be regarded as a physiological process.

LITERATURE CITED

Beck, W. A., *Plant Physiol.* (In press).

Beck, W. A., and Donnelly, Sr. M. W., THESE STUDIES, 2 (1939): 179, 259.

Beck, W. A., Schocken, K., and Donnelly, Sr. M. W., THESE STUDIES, 2 (1938): 107.

Borgström, G., Monograph, William and Norgate Ltd., London, 1939.

Crafts, A. S., *Botan. Rev.*, 5 (1939): 471.

Ruge, U. von, *Z. Botan.*, 31 (1937): 1.

Söding, H., *Jahrb. wiss. Botan.*, 79 (1933): 231.

Ursprung, A., and Blum, G., *Jahrb. wiss. Botan.*, 63 (1924): 1.

Ursprung, A., Handwörterbuch der Naturwissenschaften, Band 7, Fischer, Jena, 1932. Handbuch der biologischen Arbeitsmethoden, Abt. XI, Teil 4, Urban and Schwartzenberg, Berlin, 1937. *Cf.* p. 1111.

Went, F. W., and Thimann, K. V., Phytohormones, Monograph, Macmillan Co., New York, 1937.

Original Manuscript Submitted
April 7, 1940

A DIFFERENTIAL MICRO-RESPIROMETER

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New forms of micro-respirometers designed for measuring the oxygen consumption of small amounts of tissue have been described in the literature from time to time. In the main, these instruments were designed to meet certain requirements which could not be satisfied by available respirometers. Though some of these respirometers were designed for the special requirements of certain types of tissue, many were intended for either greater accuracy, higher sensitivity, or simplicity of manipulation.

The instrument which has hitherto been probably the most widely used for micro-respiratory measurements is the Fenn[1] differential volumeter. This volumeter has been used on several occasions by the authors. Though the sensitivity of this instrument is high enough for most work, the principal disadvantage is the difficulty of accurately controlling the index drop. Unless attention is constantly focused on it, the drop is frequently lost by passing into one of the two gas vessels. Another undesirable feature often encountered is the breaking of the index drop. When dealing with excised living tissue time is an important factor and these shortcomings are most disadvantageous. When working with human biopsies, frequently only exceedingly small fragments of tissue are obtainable.

Recently Thimann and Commoner[2] designed a differential volumeter in which control of the index drop was facilitated

[1]Fenn, W. O., The Differential Volumeter for the Measurement of Cell Respiration and Other Processes, The Science Press Printing Co., Lancaster, Pa., 1935.

[2]Thimann, K. V., and Commoner, B., *J. Gen. Physiol.*, 23 (1940): 333.

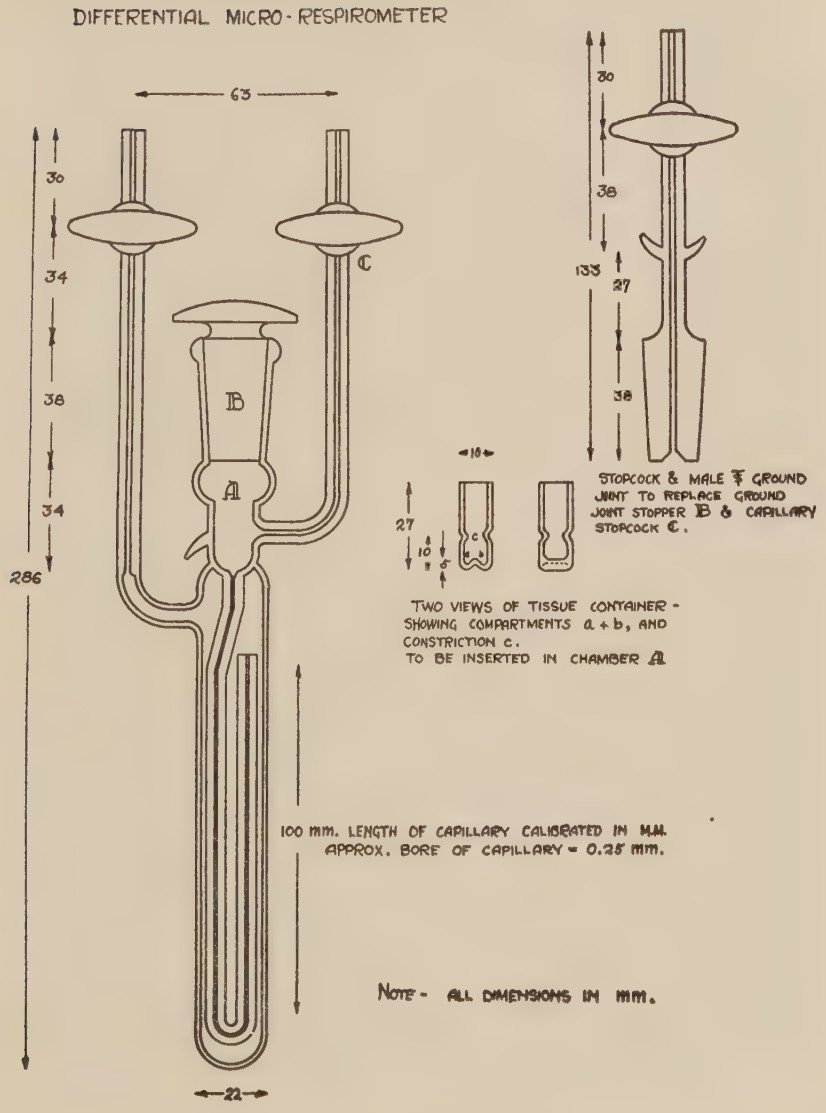
by means of a shunt capillary tube. By the use of a small experimental vessel and a large control vessel (ratio 10/1) they were able to obtain a rather high sensitivity. This instrument is composed of four separable parts: the control vessel, the reading capillary and shunt, the KOH vessel, and the experimental vessel. Although such an arrangement permits convenient and thorough cleaning of the parts, it would seem that the three joints, even when very carefully ground, would increase the possibility of leakage. However, it must be conceded that these investigators circumvented a trying obstacle by the introduction of the capillary shunt.

For some time the authors have been experimenting with the construction of various types of differential respirometers with the hope of producing eventually an instrument incorporating as many sought-for features as practicable. An attempt was made to design and construct a respirometer which could be operated in a comparatively small water bath, be of fairly high sensitivity, consist of a minimum number of demountable parts, dependably accurate, and most of all, convenient to operate. As the result of an extended series of trials these features have finally been attained, at least in major part, in the differential micro-respirometer herein described.

Description of Respirometer

As shown in Plate I and Fig. 1, the micro-respirometer is essentially a one-piece Pyrex instrument, the control and experimental chambers being permanently connected. The apparatus has only one ground joint, this being provided to insert the tissue and KOH well into the experimental chamber. The reading capillary U-tube has a bore of 0.25 mm., is calibrated in millimeters (0-100), and is sealed into the experimental chamber at one end, with the other end open to the gas space in the control chamber. Both experimental and

FIG. 1


DIFFERENTIAL MICRO-RESPIROMETER
63
30
34
38
34
286
22
A
B
C
30
38
133
27
38
10
27
10
5
STOPCOCK & MALE ₹ GROUND JOINT TO REPLACE GROUND JOINT STOPPER B & CAPILLARY STOPCOCK C.
TWO VIEWS OF TISSUE CONTAINER - SHOWING COMPARTMENTS a + b, AND CONSTRICTION c. TO BE INSERTED IN CHAMBER A
100 mm. LENGTH OF CAPILLARY CALIBRATED IN M.M.
APPROX. BORE OF CAPILLARY = 0.25 mm.
NOTE - ALL DIMENSIONS IN mm.

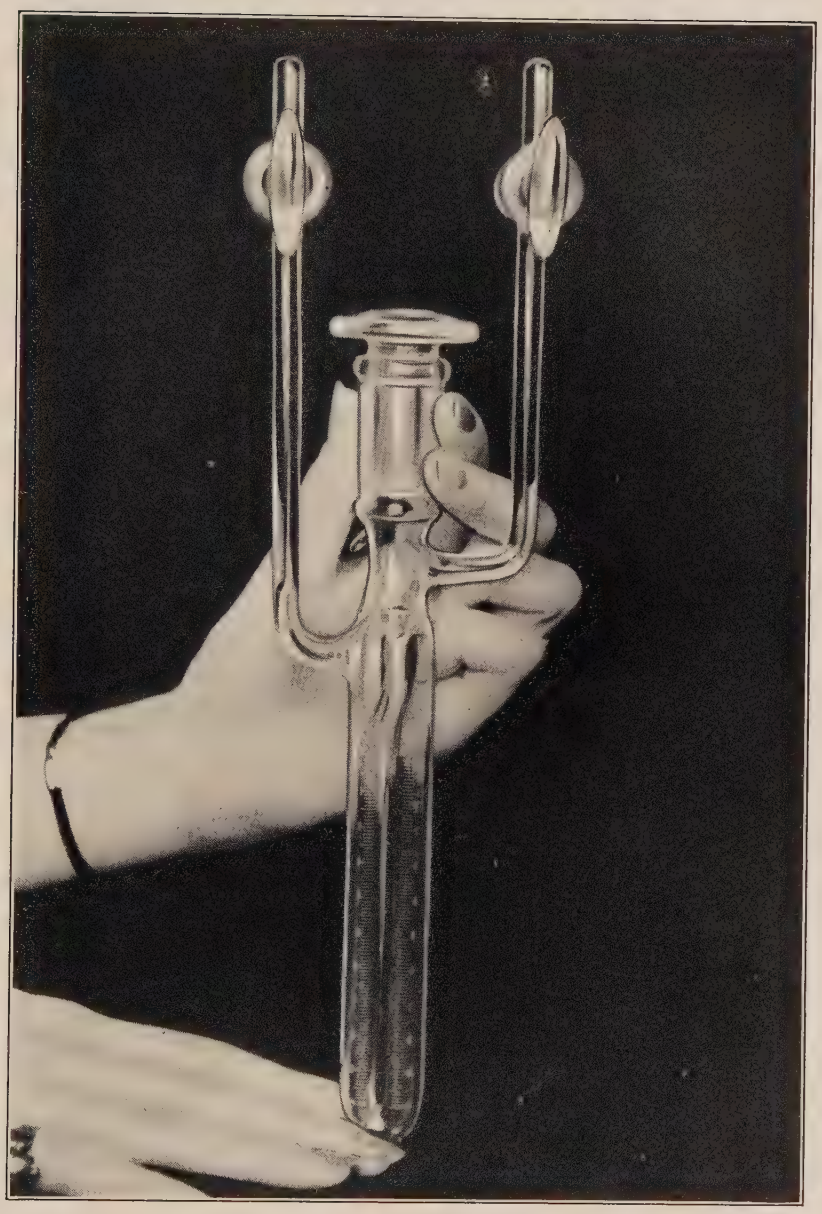
control chambers are provided with capillary stopcocks which may be opened to the outside air. In a typical respirometer which we have constructed, the experimental chamber (tissue-KOH-well inserted) has a volume of 4060 cu. mm. The volume of the control vessel is 31647 cu. mm. The ratio of the two chambers is approximately 8/1.

As shown in the diagram, the tissue well consists of a piece of glass tubing sealed at one end, which is indented, thus forming two compartments, one for the tissue and Ringer's solution, the other for tissue extracts, etc., to be tested upon the tissue. The extracts may be poured into the tissue chamber by merely tilting the respirometer. KOH paper is held above the tissue and is prevented from falling into the tissue compartment by a constriction in the glass (indicated in diagram of Fig. 1). Brodie solution is introduced into the reading capillary with a pipette inserted through the opening of the experimental chamber.

Petrolatum has been found quite satisfactory for sealing the stopper to the experimental chamber. A heavy rubber band is used, as shown in the photograph, to hold the stopper firmly in place. It is of paramount importance that the stopper accurately fit the female ground joint of the experimental chamber, for the slightest seepage of the stopper grease into the experimental chamber causes a marked drift of the manometer fluid. The authors found it necessary to regrind and repolish the standard taper ground joints with a water suspension of the finest emery flour.

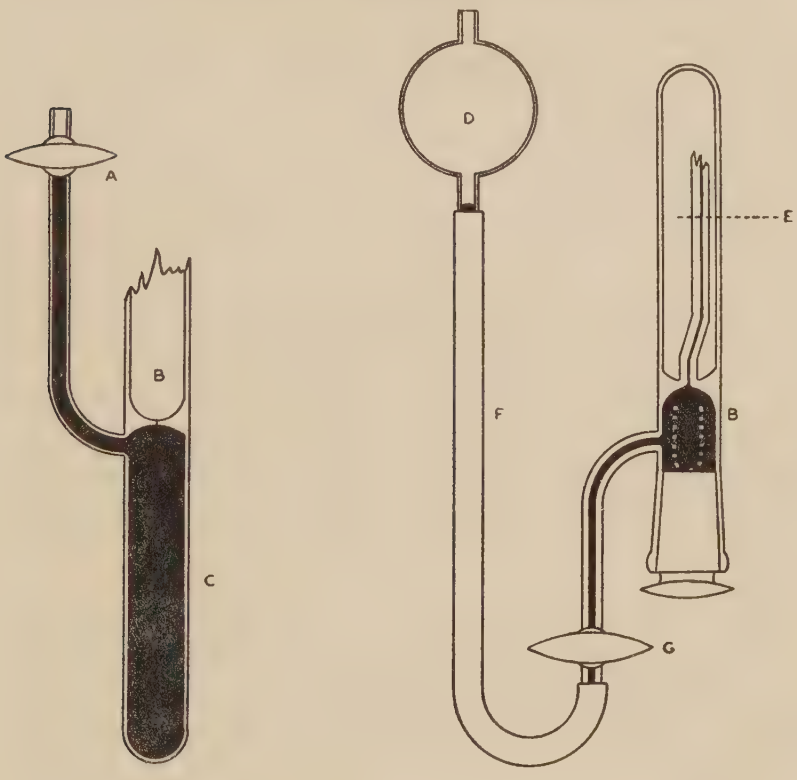
Experiments have shown that after cleaning and sterilizing the apparatus, it is only necessary to sterilize the well after each experiment. It must be borne in mind, however, that care should be taken against contamination. The chambers of the apparatus should be protected at all times from the outside air. The introduction of the tissue well into the experimental chamber and also its subsequent withdrawal after an

PLATE I



The Differential Micro-Respirometer

FIG. 2—*Calibration of Differential Micro-Respirometer*



experiment should be done with a pair of sterile forceps. When it is desirable to flush the experimental chamber with a gas, a special stopper-stopcock is provided to replace the plain stopper (shown in Fig. 1).

CALIBRATION

In order to calibrate the respirometer, the volume of the experimental and control chambers and also the volume of the reading capillary must be determined. This is done by simply filling these gas chambers with mercury.

After the respirometer has been thoroughly cleansed with nitric acid and dried in an oven, the control chamber may be filled as follows with clean mercury (*Cf.* Fig. 2): the mercury is poured into the experimental chamber (B) and is drawn through the capillary tube into the control chamber (C) by connecting stopcock (A) to a vacuum pump. After the control chamber has been completely filled (including the stopcock capillary up to the stopcock) a pointed wood applicator is pressed against the reading capillary outlet into the experimental chamber to prevent the escape of mercury from the capillary. With the stopcock closed and the applicator firmly held in place, the surplus mercury may then be poured from the experimental chamber by simply inverting the respirometer. The mercury is then poured from the respirometer into a weighing flask and the weight and temperature determined. Droplets of mercury adhering to the walls of the respirometer may be recovered by lightly tapping the instrument.

Mercury is introduced into the experimental chamber with the aid of a reservoir while the respirometer is held in an inverted position as shown at the right of the diagram in Fig. 2. Prior to filling the experimental chamber, the tissue-KOH-well is filled with mercury, placed on the stopper, and both carefully inserted into the respirometer. The broken white line in the diagram illustrates the position of the tissue-KOH-well in the experimental chamber. The mercury may then be allowed to fill the experimental chamber by raising the reservoir. Tapping the respirometer or raising and lowering the mercury will eliminate any air bubbles that might form. After the mercury has been brought into the reading capillary to the 50 mm. mark (E), the stopcock is closed. After the mercury has been withdrawn from the experimental chamber and weighed, the reading capillary is filled from the 50 mm. mark (E) to the end opening into the control chamber. This

amount is then weighed and subtracted from the amount originally introduced into the control chamber.

Since KOH-paper is used for the absorption of CO_2 in the tissue well, its displacement should be determined and subtracted from the volume of the experimental chamber. The space occupied by the KOH-paper was determined by measuring the area and thickness of the paper used (Whatman No. 40 filter paper cut into a strip 30 x 8 x 0.15 mm. and folded double).

With these measurements completed, the equation for calculating the respirometer constant may now be considered. The familiar Warburg-Barcroft equation for the differential respirometer may be expressed as follows:

$$K = \left(1 + \frac{AP}{2V_c}\right)\left(\frac{V_e \times \frac{T}{T+t}}{P} + \frac{AT}{2\,T+t}\right)$$

K = respirometer constant

V_c = volume of control chamber in cubic millimeters

V_e = volume of experimental chamber in cubic millimeters

T = absolute temperature (273°)

$P = 760 \times \frac{13.6}{D}$

D = density of capillary fluid

t = temperature of water bath

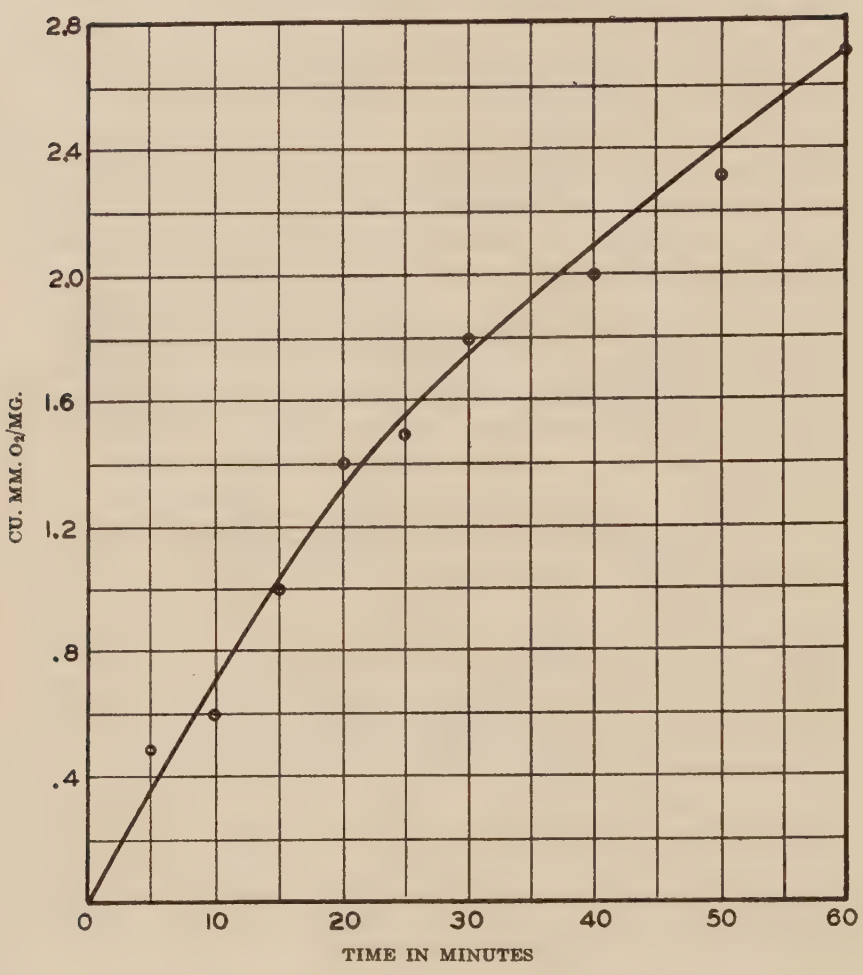
A = area of capillary bore

The oxygen uptake is then expressed as,

$$x = hK$$

in which h is the sum of the difference between the original reading of both arms of the capillary and the final reading, expressed in millimeters.

FIG. 3—*Respiration of Mouse Mammary Tissue*



The constant for the respirometer whose dimensions have been given above is derived as follows:

$$K = \left(1 + \frac{.049 \times 9856}{68294}\right)\left(\frac{4060 \times \frac{273}{273+37.5}}{9856} + \frac{.049 \times 273}{2 \times 273 + 37.5}\right) = 0.3872$$

Typical Experimental Results

Some indication of the performance and sensitivity of the micro-respirometer may be obtained from the following experiment. A small fragment of mouse mammary tissue (1.6 mg. dry weight) was excised under sterile conditions and introduced into the tissue well with 0.02 cc. Ringer's glucose-phosphate solution. About 0.05 cc. of a 5 per cent KOH solution was added to the filter paper for the absorption of the CO_2. After the respirometer had been placed in the water bath and an equilibrium period of 15 minutes allowed, the stopcocks were closed and the initial readings made. Readings were continued for an hour at 5-minute intervals. At the end of 1 hour the difference in level between the two capillary columns was 11.6 mm. The individual readings multiplied by K and divided by the dry tissue weight gave the rate of the oxygen uptake as shown in the curve of Fig. 3. The Qo_2 of mouse mammary tissue was thus found to be about 2.7. Very reliable results have been obtained with the respiration of mouse liver, the tissue fragment not exceeding 0.8 mg. in dry weight.

Summary

A micro-respirometer of the differential type was designed to meet the following requirements:

1. Ease of manipulation.
2. A minimum of separable parts.
3. A minimum displacement in the water bath.
4. A reasonably high sensitivity.

Original Manuscript Submitted
June 15, 1941.

EFFECT OF pH ON ADSORPTION BY CHARCOAL OF FACTORS INCREASING THE RESPIRATION OF YEAST AND LIVER

By ELTON S. COOK and ELSIE M. WALTER*

In an earlier paper[1] we described the effects of temperature and alcohol concentration on the adsorption of factors present in yeast extracts which increase the respiration of yeast and tissues. It was found that the most effective conditions were adsorption at the lower temperature of 5°-8° C. and elution at 60° C. Employing these conditions and using water as the solvent we have investigated the effects of pH on the adsorption.

EXPERIMENTAL

Norit charcoal was the adsorbent. Before use it was washed several times with boiling water, dried and activated by heating to redness in an iron crucible for 15 minutes.

A crude yeast extract was prepared by the previously described method[2] of treating Fleischmann's bakers' yeast with 95 per cent ethyl alcohol at 60°-70° C. for 4 hours and concentrating *in vacuo*. This extract was Fraction 1 and contained 204 mg. of solids per cc.

Two 100 cc. portions of Fraction 1 were adjusted to a pH of 2.5 with hydrochloric acid† and each was stirred well

*Assistance in the experimental work was given by Sister Mary Grace Waring, S.S.J., Sister Mary Redempta Eilert, S.S.J., and Frank J. Rack.

†All adjustments of pH in the following procedures were made with hydrochloric acid or sodium hydroxide, as required. All pH determinations were made with the glass electrode.

[1]Cook, E. S., and Walter, E. M., These Studies, 2 (1939): 189.

[2]Cook, E. S., Kreke, C. W. and Nutini, L. G., *ibid*, 2 (1938): 23.

for 5-10 minutes with 20 g. of activated Norit charcoal at 0°-5° C. The volume of each portion was increased to 250 cc. and the charcoal was filtered off and washed with an additional 50 cc. of water at pH 2.5. The filtrates and washings from both portions were combined, adjusted to pH 7, and taken to dryness in an oven at 50° C. (Fraction 2). The charcoal from one portion was suspended in 400 cc. of distilled water (normally slightly acid) at 60° C. for a few minutes and filtered. This elute was adjusted to pH 7 and taken to dryness in the oven (Fraction 3). The charcoal from the second portion was likewise suspended in 400 cc. of distilled water and the pH of the suspension was adjusted to 8.5. The suspension was heated to 60° C., filtered, and the filtrate was concentrated as before (Fraction 4).

Two 100 cc. portions of Fraction 1 were adjusted to a pH of 7 and treated with Norit charcoal as in the first case. The combined filtrates (material non-adsorbed at pH 7), after being taken to dryness, formed Fraction 5. The charcoal from one portion was suspended in 400 cc. of distilled water, the pH adjusted to 1.5, the temperature raised to 60° C., and the charcoal filtered off. The filtrate was Fraction 6. The charcoal from the other portion was treated similarly with the exception that the pH was adjusted to 8.7; the filtrate was Fraction 7. Both fractions were adjusted to pH 7 before being taken to dryness.

Lastly, two 100 cc. portions of Fraction 1 were treated similarly with Norit charcoal at pH 8.03. The combined filtrates formed Fraction 8. One portion of the charcoal was eluted with 400 cc. of water at pH 6.9 (Fraction 9) and the other with water at pH 2.5 (Fraction 10).

The weights of the various fractions are given in Table I.

The dried fractions were dissolved in distilled water at a concentration of approximately 35 mg./cc. and the pH was

TABLE I

Weights of Fractions

Fraction Number	Treatment	Weight in Grams
2	Non-adsorbed pH 2.5	41.76
3	Adsorbed pH 2.5, eluted with distilled water (slightly acid)	1.25
4	Adsorbed pH 2.5, eluted pH 8.5	2.92
5	Non-adsorbed pH 7	30.26
6*	Adsorbed pH 7, eluted pH 1.5	3.50
7	Adsorbed pH 7, eluted pH 8.7	0.90
8	Non-adsorbed pH 8	25.92
9	Adsorbed pH 8, eluted pH 6.9	1.14
10*	Adsorbed pH 8, eluted pH 2.5	1.97

*Owing to the extremes of pH and the fact that hydrochloric acid and sodium hydroxide were used to bring them to a final pH of 7, these samples contained considerable salt. They were analyzed for NaCl by titrating with silver nitrate, using dichlorofluorescein as the indicator.[3] Fraction 6 contained 83.1 per cent NaCl and Fraction 10, 49.6 per cent NaCl.

adjusted to 7.2-7.4. The solutions were filtered through a Seitz filter to remove any insoluble material and were steamed for 15 minutes before biological assay. The concentration of each sample was determined by taking 1.0 cc. of the solution to dryness in an oven at 50° C.

The fractions were assayed for their potency in increasing the oxygen uptake of rat liver and Fleischmann's bakers' yeast. These determinations were carried out in the manner described previously.[2] All assays were made at 37.5° C. and at a pH of 7.2-7.4. The medium was Ringer-phosphate-glucose (0.2 per cent glucose for tissue, 0.02 per cent glucose for yeast). Seven Warburg respirometers were employed: the first served as a thermobarometric control; the second and third as tissue or yeast controls; the fourth and fifth contained tissue or yeast

[3]Bambach, K., and Rider, T. H., *Ind. Eng. Chem., Analyt. Ed.*, 7 (1935): 165.

plus Fraction 1; and the sixth and seventh contained tissue or yeast plus the fraction to be tested. Thus, each fraction was compared directly with the parent extract (Fraction 1) on the same sample of yeast or tissue, thereby eliminating some of the error inherent in the procedure due to biological variation.

The fractions were tested on liver at concentrations between 9 and 10 mg./cc., usually at 9.36 mg./cc.; they were tested on yeast at concentrations between 0.7 and 1.3 mg./cc., usually at 0.94 mg./cc. The results of these assays are given in Table II. In this table absolute values for Qo_2 or per cent stimulation are not included but rather, for readier comparison, the per cent increase (or decrease) of stimulation given by the fraction as compared with Fraction 1. For reference it may be stated that the average oxygen consumption of the yeast (16 mg. wet weight for 25 minutes) was 58.4 cu. mm., with a range of 46.9 to 69.1 in 22 determinations. The average Qo_2 for the liver was 5.4 cu. mm./mg. (dry weight) per hour, with a range of 3.4 to 7.5 in 24 determinations. The average absolute stimulation of yeast respiration by Fraction 1 at 0.94 mg./cc. was 47 per cent, with a range of 24 to 65 per cent. The average absolute stimulation of liver respiration by Fraction 1 at 9.36 mg./cc. was 32 per cent, with a range of 23 to 50 per cent. With these figures calculations of absolute values may be made from Table II, although this is not essential for the discussion.

DISCUSSION

The effects of the fractions on liver respiration are clear-cut and consistent. The active material appears to be selectively adsorbed on activated Norit charcoal at pH 2.5 and to be eluted therefrom with distilled water (Fraction 3) or, better, with alkaline water (Fraction 4). On the other hand, the liver-

TABLE II

Percentage Increase in Oxygen Uptake of Yeast and Liver Caused by Fractions of Yeast Extract

Fraction Number	Treatment	Per Cent Increase in Respiration over *Fraction 1*	
		Yeast	Liver
2	Non-adsorbed pH 2.5.....	−34	−13
3	Adsorbed pH 2.5, eluted with distilled water (slightly acid).	+97	+261
4	Adsorbed pH 2.5, eluted pH 8.5.....	+10	+570
5	Non-adsorbed pH 7.....	+162	+104
6	Adsorbed pH 7, eluted pH 1.5.....	less active than *Fraction 1*	Depressed below normal
7	Adsorbed pH 7, eluted pH 8.7.....	−11	−28
8	Non-adsorbed pH 8.....	0	+103
9	Adsorbed pH 8, eluted pH 6.9.....	−9	−64
10	Adsorbed pH 8, eluted pH 2.5.....	0	Depressed below normal

active material is not readily adsorbed at neutral or alkaline pH, the activity of the non-adsorbed portions being doubled at these pH values (Fractions 5 and 8). The eluates from the charcoal at these pH values are less active than the parent Fraction 1 (Fractions 7 and 9) or are inhibitory to liver respiration (Fractions 6 and 10). The highly depressant action of Fractions 6 and 10 is no doubt largely due to their high salt content, pointed out earlier in this paper, and does not necessarily point to the concentration in these fractions of specific depressants.

The above facts indicate that the liver-active substance (or substances) is acid in nature. That this is true is confirmed

by experiments reported in another paper[4] in which the liver factor was concentrated by means of fractional electrical transport. In these experiments the active factor collected in the electrolytic cells between pH values 2.5 and 3. The present data give little further evidence as to the nature of the factor nor do they allow conclusion as to whether the material is acting as a stimulant in a catalytic sense or as an oxidizable substrate. The available further evidence on these points is summarized in the paper referred to.

The results are much less conclusive with regard to the yeast factor or factors. Although some of the earlier adsorption evidence could be taken as indicating the existence of a basic yeast factor,[1] this does not seem to be borne out by the present experiments. From the behavior of Fractions 2, 3, and 5 it would be concluded that the yeast factor, too, was acidic in nature and perhaps identical with the liver factor. The activity of Fraction 4, however, while qualitatively in agreement with this suggestion, falls short quantitatively. This, of course, may possibly be due to experimental errors, although the magnitude of the difference makes this seem unlikely.

It would appear that the yeast factor is somewhat indifferent to adsorption at neutral or alkaline pH, both adsorbed and non-adsorbed fractions at these pH values showing approximately the same activity as the original Fraction 1. (Results with Fractions 6 and 10 again are of questionable accuracy owing to the salt content. It may be pointed out that this fact is not so serious here inasmuch as the lower concentrations of fractions used for yeast assay reduced the salt present in the manometer flasks by about 90 per cent.) The electrolytic experiments referred to earlier gave some evidence of collection of yeast activity at acid pH, but there was no indication that the yeast and tissue active factors are identical.

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[4]Cook, E. S., Walter, E. M., Rack, F. J., Eilert, Sr. M. R., and Sawyer, M. A., This Issue, p. 147.

SUMMARY

A substance (or substances) present in aqueous-alcoholic extracts of bakers' yeast and increasing the oxygen consumption of rat liver slices appears to be acidic in nature since it is adsorbed on activated Norit charcoal at acid pH, is readily eluted by alkali, and is largely non-adsorbed at neutral or alkaline pH. The evidence with regard to the material in these extracts which increases the oxygen uptake of yeast suspensions is less clear-cut. It also appears to be acidic but is probably not identical with the liver factor.

BIBLIOGRAPHY

Bambach, K., and Rider, T. H., *Ind. Eng. Chem., Analyt. Ed.*, **7** (1935): 165.

Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

Cook, E. S., and Walter, E. M., THESE STUDIES, **2** (1939): 189.

Cook, E. S., Walter, E. M., Rack, F. J., Eilert, Sr. M. R., and Sawyer, M. A., THIS ISSUE, p. 147.

Original Manuscript Submitted
August 1, 1941.

USE OF FRACTIONAL ELECTRICAL TRANSPORT TO PREPARE A YEAST CONCENTRATE WHICH INCREASES THE OXYGEN CONSUMPTION OF LIVER*

By ELTON S. COOK, ELSIE M. WALTER, FRANK J. RACK, SISTER MARY REDEMPTA EILERT, S.S.J., and MARY ALICE SAWYER

In these laboratories we have been concerned with a study of materials from natural sources which affect cellular respiration or proliferation. Earlier work has indicated that yeast contains materials which increase the respiration of yeast and of tissues (rat liver and skin), that factors for tissues and yeast are probably distinct, and that different substances may be responsible for the respiratory effects on liver and skin.[1] The bulk of the evidence also indicates that the respiration and growth-promoting substances are distinct although they may at times appear in similar fractions,[2] and that materials other than the known bios components exist which increase the proliferation of yeast.[2,3]

Relatively crude tissue or yeast extracts undoubtedly owe their activity to a mixture of coenzyme-like and substrate materials and it is desirable to learn to what extent these various materials may be responsible for the observed effects. The present paper gives preliminary results of the application to this problem of the method of fractional electrical transport used by Williams with such success in the early studies of

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*In part from the Bradley Hall Laboratories of the Institutum Divi Thomae, Palm Beach, Florida.

[1]Cook, E. S., Kreke, C. W., and Nutini, L. G., These Studies, 2 (1938): 23.

[2]Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, 34 (1940): 1580.

[3]Williams, R. J., Eakin, R. E., and Snell, E. E., *J. Am. Chem. Soc.*, 62 (1940): 1204.

pantothenic acid.[4] By this means we have prepared a fraction having greatly enhanced ability to increase liver respiration without any increase in activity on yeast respiration. While we do not yet possess knowledge of the nature of the substance or substances involved, nor know the mode of action, i.e., whether coenzyme-like or as a substrate, it seems desirable to report our information to date while we are proceeding with the work.

EXPERIMENTAL

I. *Preliminary Experiments.*

For the first experiments several yeast extracts which were available in the laboratory were combined. These had been obtained by the extraction of Fleischmann's bakers' yeast with 95 per cent ethanol, 50 per cent ethanol, methanol and water. Previous work[1, 5] had shown that all these solvents extract the crude respiratory-stimulating factors (R.S.F.). These combined extracts were taken to dryness and dissolved in distilled water. After adjusting the pH to 7.2 with sodium hydroxide, the solution was treated with activated Norit charcoal at 60° C., as previously described,[6] and filtered. The filtrate contained unadsorbed solids in a concentration of 89.9 mg. per cc. (Fraction S-1).

The electrolysis apparatus, similar to that of Williams and Truesdail,[4] consisted of four cells connected with glass bridges. The electrodes consisted of platinum gauze supported on platinum wire. The source of current was a rectifier-transformer unit working from a 110-volt A.C. source and delivering 500 to 3000 volts D.C. In most instances the current was adjusted

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[4]Williams, R. J., Truesdail, J. H., *Ibid.*, 53 (1931): 4171; Williams, R. J., *J. Biol. Chem.*, 110 (1935): 589.

[5]Cook, E. S., and Walter, E. M., THESE STUDIES, 2 (1939): 239; and unpublished data.

[6]Cook, E. S., and Walter, E. M., *ibid.*, 2 (1939): 189.

to 2-10 milliamperes at 500-1000 volts. The electrolysis cells and bridges were sterilized by autoclaving and were enclosed in a sterile glass case which could be sterilized from time to time by a Sperti Mercolite ultraviolet lamp. Aseptic technique was used throughout since the crude fractions allow the growth of bacteria and molds. The fractions were sterilized by steaming or, occasionally, by Seitz filtration.

Five hundred cubic centimeters of sterile Fraction S-1 was divided between the cells and bridges. During the electrolysis the volume was kept constant by adding sterile distilled water as needed. An initial potential of 840 volts at 3.25 milliamperes was applied and the electrolysis was continued for 105 hours, after which time a separation was effected as shown by pH determinations of the cell contents with the glass electrode. The contents of each of the four cells were then reelectrolyzed separately, placing the acid samples in the two cells on the cathode end and the basic samples in the two cells on the anode end. The remaining cells and the bridges were filled with distilled water. The current was passed until the pH of the cell contents became practically constant, the time required being from 145 to 255 hours. In later experiments care in keeping the salt content low decreased the period of electrolysis. The resulting 17 samples are shown in Table I.

These fractions, after adjustment to a pH of 7 and a concentration of 30-40 mg./cc., were assayed for respiratory activity on Anheuser-Busch bakers' yeast, rat liver slices and rat skin by the Warburg manometric method using Ringer-phosphate-glucose medium (0.02 per cent glucose for yeast, 0.2 per cent for tissues). The techniques of assay were the same as previously described[1, 7] with the exception that in the assays on tissues, the samples to be assayed were placed in the side-arms of the manometer flasks and tipped in after

[7]Cook, E. S., and Morgan, Sr. M. N., *Biochem. J.*, 34 (1940): 15.

TABLE I

Preparation Flow-Sheet and Liver Respiratory Activity of Preliminary Fractions

		Cell No.	Fraction No.	pH	Activity on Rat Liver Respiration			
					Per Cent Increase over S-1	Concentration mg./cc.	Per Cent Increase over S-1	Concentration mg./cc.
S-1	Cell I, pH 4.65	1	S-2	2.05	−49	11.4	−43	5.7
		2	S-3	2.62	+23	12.0	+16	6.0
		3	S-4	6.90	−43	9.4	−72	4.7
		4	S-5	11.41	−58	11.6	−50	5.9
	Cell II, pH 6.35	1	S-6	1.80	−32	11.2	−83	5.6
		2	S-7	2.50	+54	10.4	+36	5.2
		3	S-8	3.80	+57	12.4	+25	6.2
		4	S-9	12.15	−41	10.8	−58	5.4
	Cell III, pH 7.00	1	S-10	2.60	+9	10.9	+24	5.5
		2	S-11	8.80	−29	12.0	−52	6.0
		3	S-12	9.60	−100	10.7	−100	5.7
		4	S-13	11.00	−100	9.1	−95	4.9
	Cell IV, pH 9.30	1	S-14	1.91	−47	11.1	0	5.6
		2	S-15	9.36	−55	10.0	−37	5.0
		3	S-16	10.81	−100	10.0	−130	4.8
		4	S-17	11.80	−100	10.1	−105	5.0

the rate of respiration of the tissues had been established. This procedure is superior in that separate controls, with different samples of tissue, were unnecessary. A lag in respiratory stimulation of approximately 15 minutes in the case of skin and 10 minutes in the case of liver was observed. Hence, these periods were deleted from the experimental respiration period in calculating results. Each fraction was compared with the original extract (S-1) on tissues from the same animal or on yeast from the same cake to eliminate as far as possible errors due to biological variation.

The data on the stimulating activity of the fractions on liver respiration are given in Table I. Here, for purposes of readier comparison, absolute stimulations are not given, but rather the increase (or decrease) in activity of the fractions over that of the parent extract S-1. The parent extract itself caused an average absolute stimulation of liver respiration of 155 per cent in a concentration of 5.7 mg./cc. It is readily seen that the liver activity collects within the pH range of 2.5-3.8. For our present purposes it is unnecessary to give the detailed data on yeast and rat skin. Suffice it to say that the skin respiratory-stimulating factor accumulated in the fractions of lowest pH (1.80 and 1.91), all others being inactive or inhibitory. Yeast stimulating activity was low, there being evidences of slight accumulation at pH 2.60 and pH 2.62.

II. *Further Experiments.*

Six hundred cubic centimeters of a concentrated extract of Anheuser-Busch bakers' yeast (S-18), prepared by alcohol extraction and Norit charcoal treatment as previously described[1, 6] and having a pH of 5.6, was electrolyzed in two portions, after which the cell contents collecting within the pH range 2.50-4.32 were combined. This material was placed in the cathode cell, distilled water being placed in the other cells, and electrolyzed until good separation was obtained as indicated by the relative constancy of pH of the cell contents. There were thus obtained four final fractions from the material which accumulated originally between pH 2.50 and pH 4.32. These fractions were: S-19, pH 2.05; S-20, pH 2.40; S-21, pH 2.54; S-22, pH 8.18.

These fractions were concentrated to approximately 30 mg./cc. and the pH was adjusted to 7. With the exception of S-22, they were assayed for their respiratory effect on rat liver, using a 15-minute equilibrium period, 20-minute period to determine rate of liver respiration, 10-minute lag period

after the addition of the sample, and a 20-minute experimental respiration period. Assays on Anheuser-Busch bakers' yeast employed a 15-minute equilibrium period and a 25-minute experimental respiration period. The results of these assays are shown in Tables II and III.

TABLE II

Rat Liver Respiratory Activity of Further Fractions

Fraction	pH of Cell Contents	Concentration mg./cc.	Qo_2*		Per Cent Stimulation	Per Cent Increase over S-18
			Without Fraction	With Fraction		
S-18	....	10	4.68	9.90	112	.....
S-19	2.05	10.1	5.03	6.02	20	−82
S-20	2.40	10.6	5.18	15.73	204	+82
S-21	2.54	9.3	4.27	16.56	286	+155

*Oxygen uptake in cu.mm./mg. liver (dry weight)/hour. All values in this table were secured on same liver.

TABLE III

Yeast Respiratory Activity of Further Fractions

Fraction	Concentration mg./cc.	Oxygen Uptake*		Per Cent Stimulation
		Without Fraction	With Fraction	
S-18	1.0	46.4	65.6	42
	0.5	44.0	52.9	20
S-19	1.0	46.4	55.1	19
	0.5	44.0	45.9	4
S-20	1.1	45.6	47.2	3
	0.51	44.0	50.8	15
S-21	0.9	45.6	59.2	30
	0.45	44.0	49.4	12
	0.09	44.0	47.5	8

*Oxygen uptake in cu.mm./16.0 mg. yeast (wet weight)/25 minutes.

DISCUSSION

It will be observed that no increase in yeast activity was obtained in any case. The most that can be said is that the activity was spread between the fractions, each being somewhat less active than S-18. On the other hand, while S-19 is less effective in increasing liver respiration than the parent S-18, there is a progressive increase in activity through Fractions S-20 and S.21. It would appear that in these experiments the liver-active substance (or substances) accumulated rather narrowly between the pH limits of 2.40-2.54 without a similar concentration of materials stimulating yeast respiration.

To determine the effect of concentration on the activity of S-21, a series of new assays was run on another liver. The concentrations in milligrams per cubic centimeter and the per cent stimulations, respectively, were: 0.096, 8 per cent; 0.96, 85 per cent; 4.8, 262 per cent; 9.6, 294 per cent.

The curve is linear up to a concentration of about 1 mg./cc. but at higher concentrations there is a flattening of the curve, very small increases being obtained by adding quantities greater than 5 mg./cc.

A third series of electrolytic fractionations was also effected on a crude yeast extract which was separated into 19 fractions by reelectrolysis, combining the methods of grouping the cell contents used in the previous sets. The final 7 fractions were assayed only on liver and, to conserve space, need not be reported in detail. They confirmed the previous general findings, with the greatest activity concentrating in the cells at pH 2.94-2.98. These fractions, giving about 200 per cent stimulation at 5.3 mg./cc., were not quite as active as those in the preceding set, but the pH values may be somewhat more accurate since the fractionation was more extensive.

In confirmation of the acidic nature of the active material, experiments on charcoal adsorption were carried out. These

are reported in detail in an accompanying paper[8] but they may be summarized here. A crude yeast concentrate similar to those previously employed was divided into portions, the pH values of which were adjusted to 2.5, 7.0 and 8.0 respectively. These were treated with activated Norit charcoal at 5° C. Separate portions of the charcoal from the treatment at pH 2.5 were eluted with distilled water and with water at pH 8.5; those at pH 7.0 were eluted with water at pH 1.5 and 8.7; and those at pH 8.0 were eluted with water at pH 2.5 and 6.9 (pH adjustments with sodium hydroxide or hydrochloric acid; elutions at 60° C.). These eluates, together with the corresponding unadsorbed materials, were assayed for their respiratory activity on rat liver. The order of activity was exactly as would be expected. The greatest activity was shown by the material adsorbed at pH 2.5 and eluted at pH 8.5 (154 per cent stimulation as compared with 30 per cent stimulation by the original crude at 9.36 mg./cc.). The material was not adsorbed appreciably at pH 7 or higher, the unadsorbed fractions at these pH values showing doubled activity.

The active material appears not to be amphoteric (or at the most only weakly basic) since an active sample placed in the anode cell at a pH of 1.5 did not migrate during 137 hours of electrolysis and, indeed, was increased in activity. This fact, plus the pH at which the material accumulates, helps to eliminate the amino acids from consideration as the active material although, of course, it is by no means conclusive. This is of some importance since many amino acids are oxidized by liver slices *in vitro*.[9] Most of the amino acids tested are of a low order of activity in increasing the respiration of bakers' yeast.[10]

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[8]Cook, E. S., and Walter, E. M., THIS ISSUE, p. 139.

[9]Miyao, S., *Gann.*, **29** (1935): 10; *Chem. Abst.*, **29** (1935): 5181; Kisch, B., *Biochem. Z.*, **238** (1931): 351; **242** (1931): 26; **244** (1932): 451; **247** (1932): 365.

[10]Cook, E. S., Walter, E. M., and Eilert, Sr. M. R., *Proc. Soc. Exptl. Biol. Med.*, **44** (1940): 547.

The material is inactivated by refluxing for one-half hour with methanol in the presence of 0.5 N sulfuric acid and is partially to completely inactivated by similar treatment at 30° C. It cannot be stated with certainty that this inactivation is due to esterification, since attempts at regeneration by hydrolysis are as yet inconclusive.

The material is stable to steaming at a pH of 4.5 and will withstand autoclaving at a similar pH with only slight loss. It is not equally stable to autoclaving where the pH is slightly alkaline. Seitz or Berkefeld filtration may be attended by slight losses in activity.

The findings thus far reported suggest some similarity to the properties of pantothenic acid which is rather richly present in bakers' yeast[11] but which collects at the higher pH of 3.6-3.8 during fractional electrolysis. Tests with calcium (+) pantothenate* showed it to be inactive on rat liver respiration. It is also essentially inactive on the respiration of unstarved Fleischmann's[12] or Anheuser-Busch bakers' yeast, as is its β-alanine component.[10] This is probably due to the facts that such yeast possesses an optimal or near optimal pantothenic acid content and that liver is one of its richest sources.[13] We also found cozymase* to be inactive on rat liver respiration.

Fractions S-1, S-8 and S-21 were assayed for their effect on the proliferation of a pure strain of *Saccharomyces cerevisiae*, isolated from Fleischmann's bakers' yeast, in Reader's medium by the technique previously used by us.[2] Fraction S-1 was inactive, while S-8 showed negligible, and S-21 only slight

*Kindly furnished by Merck and Co., Inc.

[11]Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135** (1940): 213.

[12]Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, **22** (1939): 637.

[13]Rohrmann, E., Burget, G. E., and Williams, R. J., *Proc. Soc. Exptl. Biol. Med.*, **32** (1934): 473; Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Nutrition*, **18** (1939): 247; Jukes, T. H., *ibid.*, **21** (1941): 193.

growth activity, whereas calcium (+) pantothenate showed a considerably higher growth activity under these conditions.† These growth experiments are being extended. Although the fractions are obviously impure, the facts cited above, especially with regard to respiratory activity, seem definitely to differentiate the material from pantothenic acid.

Ultraviolet absorption spectra (at pH 7.2) were taken of several of the fractions obtained in the preliminary experiments. While the original extract (S-1) showed distinct absorption at 2600 Å, this selective absorption disappeared with the liver-active fractions, such as S-3 and S-8, which showed essentially end-absorption. In the past we have not been able to correlate ultraviolet absorption at 2600 Å with respiratory activity of yeast fractions on yeast or rat skin.[6] Such absorption has characterized crude proliferation-promoting factors from injured cells (yeast and tissues[14]) but not any of the known bios components.[2]

The active fractions, in distilled water solutions of from 1 to 3 per cent concentration, were strongly dextrorotatory. However, some of the inactive or slightly active fractions also exhibited dextrorotation and it is unwise at this time to attempt to relate biological to optical activity in these impure fractions beyond pointing out that the biologically active fractions possess this property. Although biologically inactive materials no doubt may contribute to the optical activity of the present fractions, work in progress leads the authors to suspect that the biologically potent materials are optically active.

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†We are indebted to Sister Ann Gertrude Cronin for carrying out these assays.

[14]Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° Congr. Intern. Chem.*, **5** (1939): 26; Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, **142** (1938): 573; Loofbourow, J. R., Dwyer, Sr. C. M., and Lane, Sr. M. M., *Biochem. J.*, **34** (1940): 432.

It may be observed that the active fractions gave a positive Molisch reaction with negative Benedict, Bial and biuret reactions, contained nitrogen and phosphorus, but no sulfur, and fluoresced blue under ultraviolet illumination. It is obviously impossible, at the present time, to say to what extent these facts are to be correlated with the biological activity. For example, it cannot be stated whether the apparent presence of carbohydrate is adventitious, although the non-migration of carbohydrate under the conditions of fractionation and the absence of ability to reduce Benedict solution suggest that it might be present in some combined form. It may be recalled, parenthetically, that the respiration assays were carried out in the presence of glucose and that the respiration of liver slices does not appear to be affected by changes in concentration of glucose. It should, of course, be borne in mind that the material responsible for the observed increase in oxygen uptake may be acting as an oxidizable substrate rather than in a catalytic manner. Further studies are in progress in an attempt to elucidate these problems.

SUMMARY

Subjection of an alcoholic, non-charcoal adsorbed (at pH 7.2) yeast extract to high voltage fractional electrical transport brings about the concentration of a material which increases the oxygen uptake of rat liver but which shows little activity on the respiration or growth of yeast. The active material collects in greatest concentration at pH 2.5-3.0. Its charcoal adsorption behavior accords with its acidic nature. The active material appears to be distinct from pantothenic acid and to show no selective ultraviolet absorption.

LITERATURE CITED

Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, **34** (1940): 1580.

Cook, E. S., Kreke, C. W., and Nutini, L. G., THESE STUDIES, **2** (1938): 23.

Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° Congr. Intern. Chem.*, **5** (1939): 26.

Cook, E. S., and Walter, E. M., THESE STUDIES, **2** (1939): 189, 239, THIS ISSUE, p. 139.

Cook, E. S., Walter, E. M., and Eilert, Sr. M. R., *Proc. Soc. Exptl. Biol. Med.* **44** (1940): 547.

Jukes, T. H., *J. Nutrition*, **21** (1941): 193.

Kisch, B., *Biochem. Z.*, **238** (1931): 351; **242** (1931): 26; **244** (1932): 451; **247** (1932): 365.

Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, **142** (1938): 573.

Loofbourow, J. R., Dwyer, Sr. C. M., and Lane, Sr. M. M., *Biochem. J.*, **34** (1940): 432.

Miyao, S., *Gann.*, **29** (1935): 10; *Chem. Abst.*, **29** (1935): 5181.

Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135** (1940): 213.

Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, **22** (1939): 637.

Rohrmann, E., Burget, G. E., and Williams, R. J., *Proc. Soc. Exptl. Biol. Med.*, **32** (1934): 473.

Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Nutrition*, **18** (1939): 247.

Williams, R. J., Truesdail, J. H., *J. Am. Chem. Soc.*, **53** (1931): 4171.

Williams, R. J., *J. Biol. Chem.*, **110** (1935): 589.

Williams, R. J., Eakin, R. E., and Snell, E. E., *J. Am. Chem. Soc.*, **62** (1940): 1204.

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DESTRUCTION OF MOLD SPORES BY IRRADIATION

By SISTER M. PETRONELLA SCHROEDER, C.PP.S.

With the general recognition of the practical value of selected ultraviolet radiations, there have been developed various types of mercury arcs emitting restricted portions of the spectrum which are bactericidal and fungicidal.

The relative bactericidal actions of the various portions of the spectrum as cited in the literature are quite varied.[1, 2] Burger[3] reports an interesting study made on the destruction of bacteria by projecting the spectrum of a quartz mercury arc on bacterial-coated agar-covered slides. After a short period of incubation the agar-covered slides presented a definite series of transparent lines corresponding to definite regions of the spectrum. These transparent lines indicated the regions in which bacteria had been killed. Although maximum killing was found at 2800 Å, 2650-2700 Å, and 2540 Å, the experiments verified the findings of Sperti and Schneider[4] that all wavelengths shorter than approximately 3000 Å were effective in the destruction of microorganisms.

Fulton and Coblentz,[5] investigating the fungicidal properties of ultraviolet, succeeded in killing *Rhizopus nigricans*, propagated to maturity, by 1-minute exposure to radiations from a

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[1]Newcomer, H. S., *J. Exptl. Med.*, **26** (1917): 841; Browning, C. H., and Russ, S., *Proc. Roy. Soc. (London) B.*, **90** (1917): 33; Cernovodeanu, P., and Henri, V., *Comp. rend.*, **150** (1910): 549; Mashimo, T., *Mem. Coll. Sci. Kyoto Imp. Univ.*, **4** (1919): 1; Bayne-Jones, S., and Lindner, P., *Bull. Johns Hopkins Hosp.*, **34** (1923): 11.

[2]Coblentz, W. W., and Fulton, H. R., *Scientific Paper* **495**, *U. S. Bureau Standards* (1924).

[3]Burger, G. N., *Bull. Basic Sci. Research*, **2** (1928): 55.

[4]Sperti, G., and Schneider, H., *Bull. Basic Sci. Research*, **1** (1926): 7.

[5]Fulton, H. R., and Coblentz, W. W., *J. Agr. Research*, **38** (1929): 159.

110-volt quartz mercury arc lamp consuming approximately 320 watts in the burner.

Welch[6] reported studies on the effects of ultraviolet light on molds. He used a "C" carbon lamp, operating at 60 amperes and 50 volts, without screens or reflectors. Exposure for 1 minute at a distance of 10 inches destroyed more than 90 per cent of the molds investigated.

Read[7] made a comparison of the fungicidal potency of radiations from three models of the mercury vapor lamp with the open C_4 type of carbon lamp without reflectors or screens. The wattages of the three were 450, 475, and 1100 respectively. According to his report the ultraviolet intensity at a distance of 6 inches, of the first two lamps, was not sufficient to inactivate the spores in a short enough time to show any commercial promise. The third model, at the same distance, proved to be quite destructive to mature spores. Exposures for 1 minute at this distance generally caused complete destruction of the spores of *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor*.

Comparison of Ultraviolet Sources

Since the time of Read's investigation, simplified and inexpensive enclosed mercury arcs have been developed. These new arcs are available in different types of ultraviolet-transmitting glass as well as at different operating mercury vapor pressures.

The present experiments are a study of the effect of radiations by several models of these arcs to determine their relative efficiencies in controlling the growth of mold.

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[6]Welch, H., *J. Prevent. Med.*, **4** (1930): 295.

[7]Read, J. W., *Cereal Chem.*, **11** (1934): 80.

PLATE I

Radiation Effect of Three Mercury Vapor Arcs on Molds

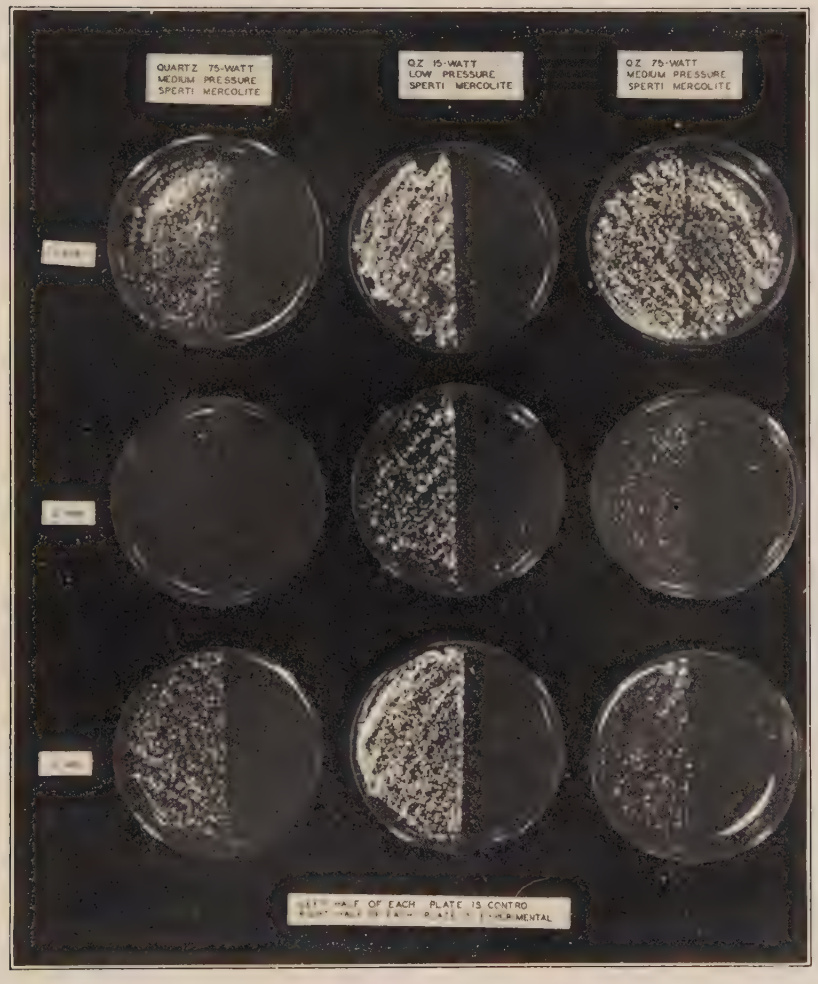


PLATE II

Comparison of Spectrograms of 75-Watt Quartz Medium Pressure, 15-Watt QZ Low Pressure, and 75-Watt QZ Medium Pressure Mercury Vapor Arcs at 40 Centimeters

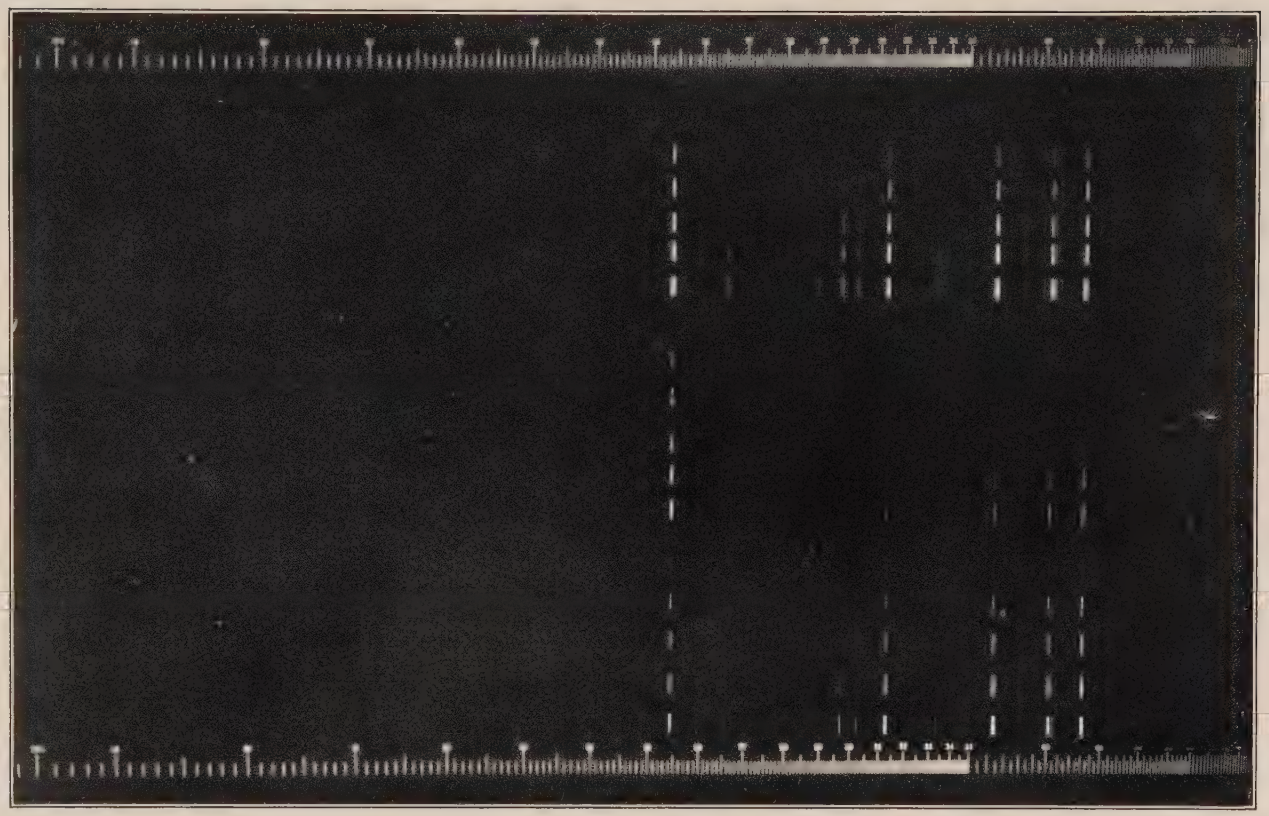


PLATE III

Spectrogram of 75-Watt QZ Medium Pressure Mercury Vapor Arc

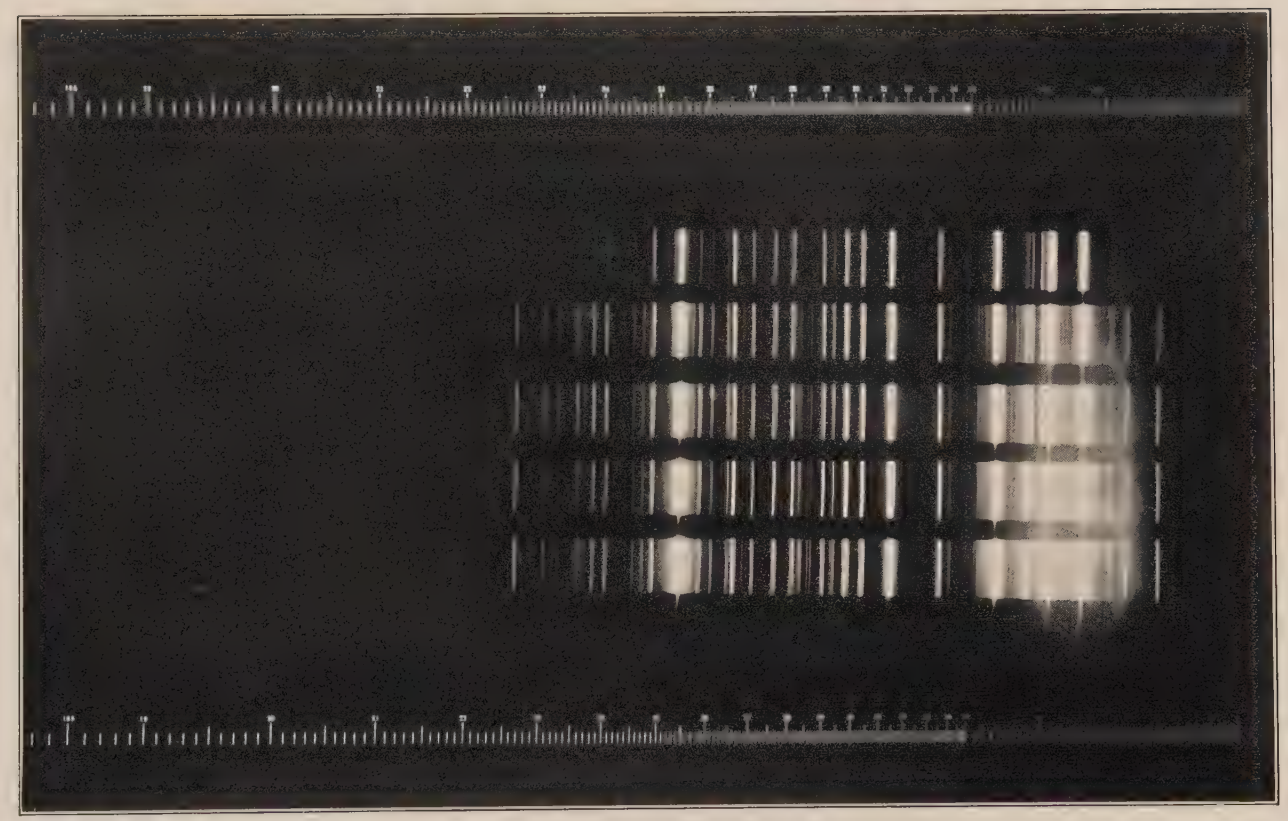
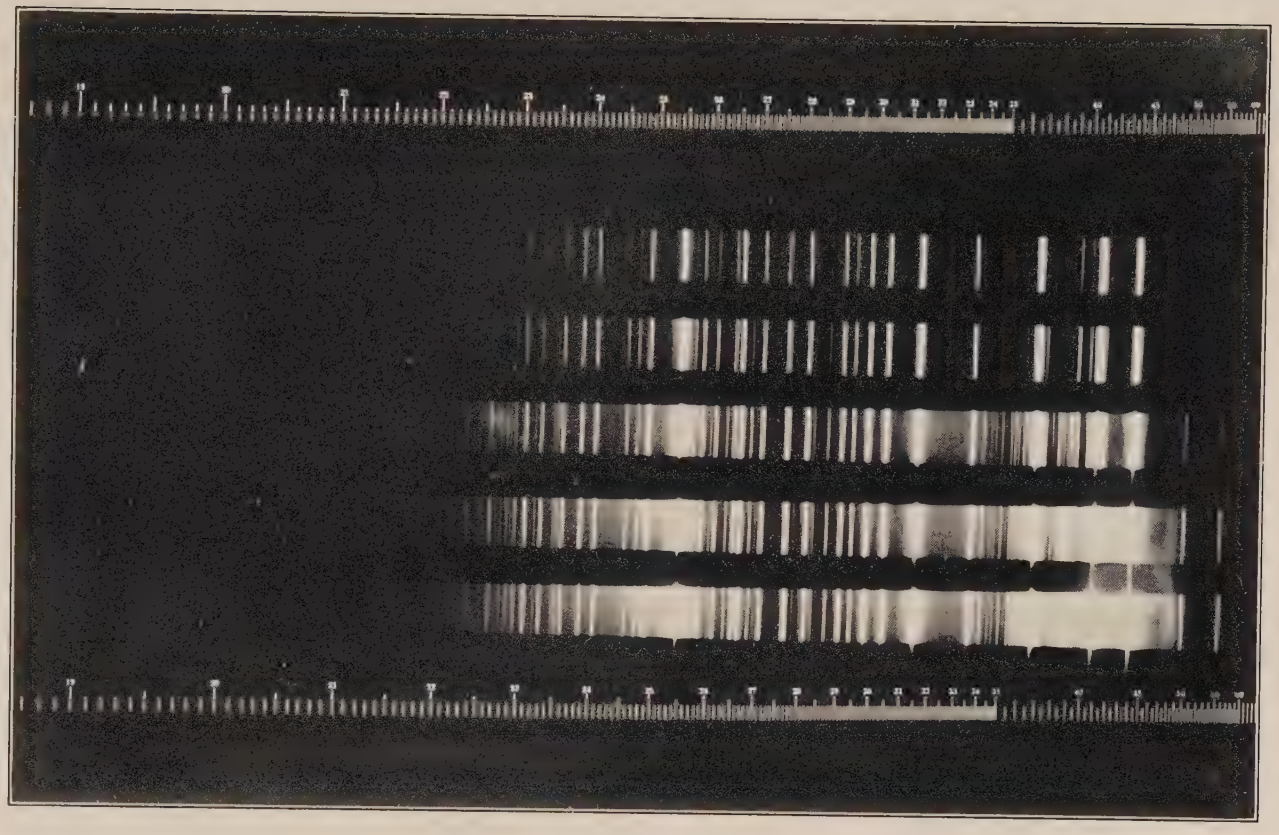


PLATE IV

Spectrogram of 75-Watt Quartz Medium Pressure Mercury Vapor Arc



Experimental

The lamps used in the experiments were: Quartz 75-watt medium pressure mercolite; QZ 15-watt low pressure mercolite; QZ 75-watt medium pressure mercolite.*

Sabouraud's medium in petri dishes was used for the culturing of a mixture of several species of mold obtained from a typical moldy loaf of bread. Two were identified, *Aspergillus niger* and *Rhizopus nigricans.*

In preliminary tests it was found that exposure of the standard Sabouraud's medium to irradiation of the intensities and for the maximum period of exposure used in these investigations had no effect upon the subsequently seeded medium. This is in agreement with the findings of Coblentz and Fulton[2] with regard to the effect of short periods of radiation on media of various composition.

Inoculations were made by transferring one loopful of an aqueous suspension of spores, which were uniformly distributed over the entire surface of the medium by means of sterile cotton swabs.

Half of each petri plate thus seeded was covered by a metal shield and so served as a control. Immediately after the plates had been seeded with spores, they were irradiated for different periods of time, at a distance of 6 inches from the surface of the medium. Growth of both irradiated and non-irradiated spores was tested by subsequent incubation of the plates for 48 hours at 37° C.

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*These lamps were manufactured and supplied by Sperti, Inc., Cincinnati, Ohio. QZ is the designation given by the manufacturers to a special type of ultraviolet transmitting glass. This glass has strong transmission up to and including the 2540 Å mercury line.

RESULTS AND DISCUSSION

Plate I presents typical results obtained by radiations from the three arcs used in the experiments. The white flecks are the measure of the number of spores which survived irradiation.

To aid in following this discussion of the spectral properties of the investigated mercury arcs as they are referred to in the paragraphs which follow, the spectrograms of the lamps are given in Plate II. These spectrograms were obtained with a Hilger quartz spectrograph at 1-, 2-, 4-, 8- and 16-second exposures, the lamps being placed at a distance of 40 cm. from the slit of the spectrograph with a slit width of .09 mm. In Plates III and IV are shown prolonged exposures of the 75-watt arc, one in QZ glass (Plate III) and one in quartz (Plate IV) in which the energy shorter than 2537 Å can be better compared.

Quartz 75-Watt Medium Pressure Mercolite

Irradiation by the quartz 75-watt medium pressure mercury arc produced 100 per cent spore destruction on each of the plates exposed for 1, 2, and 3 minutes respectively (Plate I, Column 1).

Although this lamp shows great killing power, for practical purposes quartz lamps generally are too expensive. Moreover, the nauseating effect from the ozone generated by this lamp together with the harmful effect of its short radiations make its use undesirable in the vicinity of workers. In addition, the harmful effects of ozone and the short radiations on food products make this lamp objectionable for use in the irradiation of foods. Aside from the fact that ozone causes a disagreeable odor, experimental evidence attests that it is not as powerful a sterilizing agent as it once was thought to be. The unpublished results of Loofbourow[8] show in the case of

[8]Loofbourow, J. R., Unpublished Researches.

the mold *Aspergillus glaucus* a 100 per cent destruction only after 14 to 15 minutes in a highly concentrated atmosphere of this gas. Tests for ozone carried out on the three arcs used in this investigation, at the position of the seeded plates during the longest irradiation period, were all negative. Tests for this same gas above the burning tubes were definitely positive in the case of the two 75-watt arcs. This would seem to indicate that the ozone formed was carried upward in the convection currents created by the heat given off by the lamps, and was not even measurable below the lamps at the location at which the spores were exposed to the radiations. Furthermore, it would seem to point to the fact that the action of the light is exerted directly upon the organisms and not through the formation of some toxic substance surrounding the medium.

QZ 75-Watt Medium Pressure Mercolite

The cultures shown in Plate I, third column, illustrate the effects produced by the QZ mercury arc of the same operating potential as the one enclosed in the quartz tube. Approximately 90 per cent killing was effected after irradiation for 3 minutes, the longest period under consideration in the experiments performed.

If the spectrogram of this arc (Plate III) is compared with that of the quartz 75-watt arc (Plate IV), it will be noted that the intensity of radiation of wavelength 2537 Å is notably less in the QZ arc.

The effectiveness of the quartz lamp evidently could not be attributed chiefly to the radiations of longer wavelengths since these are more intense in the lamp by which less killing was produced.

QZ 15-Watt Low Pressure Mercolite

Inhibition of the growth of spores by radiations from this arc are shown in Plate I, second column. The luxuriant growth on the left half of each plate, after 48 hours incubation at

37° C., gives an idea how heavily the plates had been seeded with spores previous to irradiation. The scarcity of white flecks on the right half of each plate indicates how very few spores survived irradiation for the several experimental time periods.

This lamp does not seem to produce a great deal of energy in the region shorter than 2537 Å. Consequently the nauseating effects of ozone are greatly minimized.

If considered from the standpoint of fungicidal effectiveness alone, this lamp would be the most desirable for the destruction of mold spores.

When the killing power of the three lamps is studied along with their spectral distribution, it is apparent that the quartz mercury arc, which shows the highest killing, has the most energy in the 2537 Å region and shorter. The 15-watt QZ lamp, which is next in killing power, also is second in intensity of the 2537 Å line; and the 75-watt QZ arc is lowest in its killing power and has the weakest emission at 2537 Å. The differences in emission at 2537 Å are attributable to: (1) the type of glass of which the arc envelope is made; (2) the pressure of the mercury vapor in the lamp.

The reason the 75-watt QZ lamp shows a weaker emission than the 75-watt quartz in the 2537 Å region is due to the filtering action of the QZ glass. The reason the 75-watt QZ is weaker than the 15-watt QZ in the same region is because the former operates at a much higher current density and pressure. The killing action cannot be attributed to the energy in the longer wavelength regions for the lamp with the most energy in this region is the lamp which shows the least killing power. The high killing power at 2537 Å is in accordance with known facts.

If considered from the standpoint of fungicidal effectiveness alone, the 15-watt QZ arc (which has most of its ultraviolet energy concentrated in the 2537 Å line) would be the most

desirable. However, if food products are to be irradiated or persons exposed to the rays, ultraviolet sources with a minimum amount of energy in the 2537 Å region are preferable.

Rider *et al.*[9] in their excellent review discuss the rapid destruction of vitamin D which takes place when the vitamin is exposed to the shorter ultraviolet rays (including the 2537 Å line).

The harmful effects on the eyes of rays shorter than those present in the sun's spectrum (shorter than 2900 Å) is well known. That they are contraindicated in diseases such as pulmonary tuberculosis is also well established.

It is apparent therefore that the selection of the type of ultraviolet source is dependent on the type of material to be irradiated and the conditions under which the irradiation is to be carried out.

It has been shown by Sperti[10] that if food products in general are irradiated, it is necessary, in order to preserve the flavor and prevent the production of malodors, to filter out all radiations shorter than approximately 2800 Å. Norris[11] has shown that in order to prevent the destruction of certain enzymes the shorter wavelengths must be excluded. In fact, all the enzymes he has tested are destroyed by the short ultraviolet rays.

SUMMARY

Assuming the 2-minute exposure of the 75-watt QZ lamp to be equal to the 1-minute exposure of the 15-watt low pressure lamp, it seems safe to say that the low pressure lamp has at least ten times the fungicidal potency of the medium pressure lamp when the two arcs are enclosed in a glass such

[9]Rider, T. H., Sperti, G., Goode, G. P., and Cassidy, H. G., *J. Am. Med. Assoc.*, **106** (1936): 425.

[10]Sperti, G., U. S. Patent Nos. 1,676,579 and 1,982,029.

[11]Norris, R. J., *Bull. Basic Sci. Research*, **2** (1928): 89.

as QZ. It is difficult to compare the quartz lamp with the exposures here given since the lowest exposure of the 75-watt medium pressure quartz lamp gives complete killing and therefore the range of exposures is not adequate.

If foods are to be sterilized, as a rule, sources with high emission in the 2800-2900 Å region and relatively low emission in the shorter regions are preferred.

In view of the many known harmful effects of short ultraviolet rays, it is apparent that where persons are exposed during the process of sterilization of food products or air, an attempt should be made to eliminate as far as possible radiations shorter than 2800 to 2900 Å. If the radiations are being used for the sterilization of such items as glassware and utensils and if no persons are exposed to the rays, then the shorter radiations are preferable because of the increased fungicidal efficiency.

LITERATURE CITED

Bayne-Jones, S., and Lindner, P., *Bull. Johns Hopkins Hosp.*, **34** (1923): 11.

Browning, C. H., and Russ, S., *Proc. Roy. Soc. (London) B.*, **90** (1917): 33.

Burger, G. N., *Bull. Basic Sci. Research*, **2** (1928): 55.

Cernovodeanu, P., and Henri, V., *Comp. rend.*, **150** (1910): 549.

Coblentz, W. W., and Fulton, H. R., *Scientific Paper* 495, *U. S. Bureau Standards* (1924).

Fulton, H. R., and Coblentz, W. W., *J. Agr. Research*, **38** (1929): 159.

Mashimo, T., *Mem. Coll. Sci. Kyoto Imp. Univ.*, **4** (1919): 1.

Newcomer, H. S., *J. Exptl. Med.*, **26** (1917): 841.

Norris, R. J., *Bull. Basic Sci. Research*, **2** (1928): 89.

Read, J. W., *Cereal Chem.*, **11** (1934): 80.

Rider, T. H., Sperti, G., Goode, G. P., and Cassidy, H. G., *J. Am. Med. Assoc.*, **106** (1936): 425.

Sperti, G., U. S. Patent Nos. 1,676,579 and 1,982,029.

Sperti, G., and Schneider, H., *Bull. Basic Sci. Research*, **1** (1926): 7.

Welch, H., *J. Prevent. Med.*, **4** (1930): 295.

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ULTRAVIOLET ABSORPTION OF SOME ORGANIC COMPOUNDS*

Part I. Aliphatic Compounds

By SISTER MIRIAM MICHAEL STIMSON, O.P., and SISTER MARY AGNITA REUTER, O.P.†

In order to provide easily accessible absorption data on the more common organic compounds a study of the literature of the near ultraviolet has been undertaken and the findings have all been compared on the basis of wavelength in Ångstroms and logarithms of the molecular extinction coefficient.

As regards absorption in the near ultraviolet (2000-4000Å), the principal absorbing groups are those involving resonators[1] or chromophores,[2] as they are sometimes called. Such absorbing groups are chiefly:

$$\rangle C{=}C\langle,\ \rangle C{=}N-,\ \rangle C{=}O,\ -N{=}N-,\ -N{=}O,\ \rangle C{=}S,\ \rangle S{=}O$$

either in simple form or in combinations. When such resonators are separated by a non-resonator the effect of the individual resonator is independent of the others and the resulting absorption curve is the sum of the absorptions of the individuals and the arrangement is said to be "coördinatively unsaturated."[3] Replacement of one group by another of similar type but of

*This review covers data obtained since 1920.

†From the Siena Heights College Unit of the Institutum Divi Thomae, Adrian, Michigan.

[1]Brode, W. R., Chemical Spectroscopy, Wiley and Sons, 1939, New York, pp. 124-140.

[2]Dutt, S., *J. Chem. Soc.*, (1926): 1171; *J. Indian Chem. Soc.*, 4 (1927): 99.

[3]Dilthey, W., *Ber.*, 53 (1920): 261; Hausser, K., Kuhn, R., and Seitz, G., *Z. physik. Chem.*, B. 29 (1935): 391.

greater mass has the tendency to shift the spectrum to longer wavelengths (decrease the frequency of vibration) and to increase the absorption.[4] However, the specific location and intensity of a chromophore is influenced by a number of factors.

For a review of methods in ultraviolet absorption spectroscopy the reader is referred to Brode[1] and Miller,[5] and for the theories of color to Lewis and Calvin.[6]

Hydrocarbons

Saturated hydrocarbons, either in a straight chain or cyclic, are not absorbent in the near ultraviolet. In the case of ethane, Scheibe and Grieneison[7] found end absorption beginning at about 1600 Å, and Carr and Stücklen[8] found that absorption for hexane and heptane began at wavelengths shorter than 1530 Å. Working with hexane, Bradley and Richardson[9] found end absorption to begin about 2500 Å and to increase very slowly, while cyclohexane absorbed beginning about 2800 Å. In no case, however, was the slope of the absorption curve very steep.

Unsaturated hydrocarbons show the first band at a wavelength less than 1530 Å, and ethylene shows further absorption between 1650 Å and 1980 Å.[7] In a broader study of simple olefin hydrocarbons, Carr and Stücklen[8, 10] found that the spectra of different hydrocarbons having the same arrangement of alkyl groups around the double bonds are almost

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[4]Hausser, K., Kuhn, R., and Smakula, A., *Ibid.*, B. 29 (1935): 384.

[5]Miller, E. S., Quantitative Biological Spectroscopy, 2nd edition, 1940, Burgess Publishing Company, Minneapolis, Minnesota.

[6]Lewis, G. N., and Calvin, M., *Chem. Rev.* 25 (1939): 273.

[7]Scheibe, G., and Grieneison, H., *Z. physik. Chem.*, B. 25 (1934): 52.

[8]Carr, E. P., and Stücklen, H., *Ibid.*, B. 25 (1934): 57.

[9]Bradley, T. E., and Richardson, D., *Ind. Eng. Chem.*, 32 (1940): 963.

[10]Carr, E. P., and Walker, M. K., *J. Chem. Phys.*, 4 (1936): 751.

identical. The position of the first absorption band is determined by the number of hydrogen atoms bound to the carbon atoms of the double bond and is practically independent of the nature of the alkyl substituents. It is shifted progressively toward the visible as the number of alkyl groups increases.

In studying the position of $\rangle C=C\langle$ absorption in six purely hydrocarbon dienes, Booker, Evans and Gillam[11] found the maximum to lie between 2170 and 2270 Å. E was as high as 35,000 but was usually between 8000-20,000. Scheibe[12] found that cyclization caused a slight lowering of the peak with a shift to longer wavelengths, which finding was confirmed by Bradley and Richardson,[9] who pointed out that cyclization may give the appearance of increased unsaturation, where this does not exist. In general it may be concluded[13] that the $\rangle C=C\langle$ chromophore absorption shifts from 1850 to 2200 Å on conjugation.

Halogen Derivatives

Weighting of saturated hydrocarbons by means of substitution with halogen causes a shift of the absorption band to the near ultraviolet. Marvel, Sample and Ray[14] found steep absorption for dichloropentanes below 2400 Å but they were not able to locate the maximum. Using a vacuum spectrograph, Herzberg and Scheibe[15] found that methyl chloride absorbs between 1530-1930 Å with a maximum at about 1750 Å. Substitution of bromine or iodine for the chlorine

[11]Booker, H., Evans, L. K., and Gillam, A. E., *J. Chem. Soc.*, (1940): 1453.

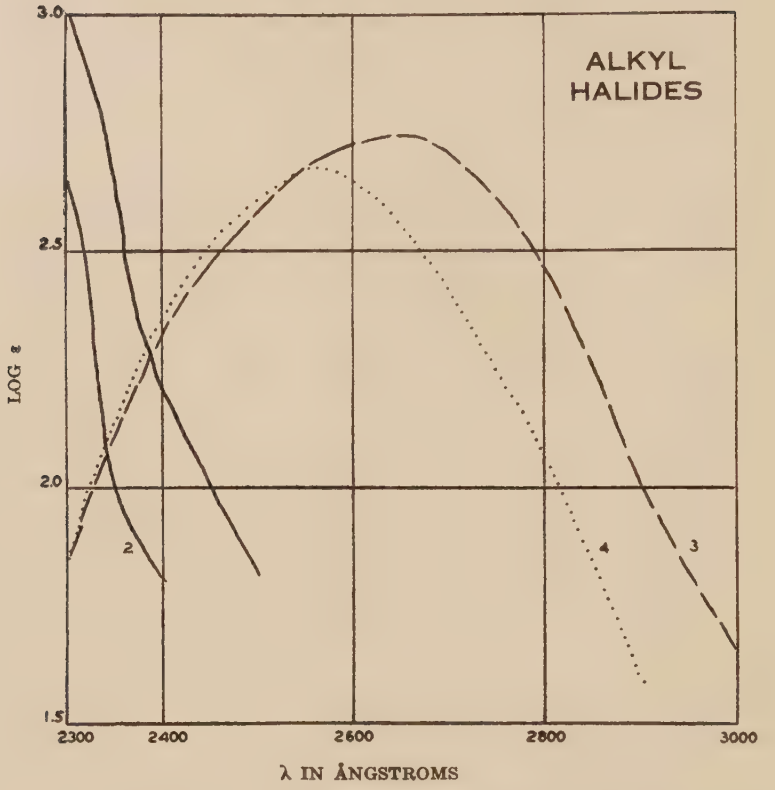
[12]Scheibe, G., *Ber.*, 59 (1926): 1333.

[13]Ausmüller, W., Fromherz, H., and Strother, C., *Z. physik. Chem.*, B. 37 (1937): 30.

[14]Marvel, C. S., Sample, J. H., and Ray, M. F., *J. Am. Chem. Soc.*, 61 (1939): 3241.

[15]Herzberg, G., and Scheibe, G., *Z. physik. Chem.*, B. (1940): 390.

FIG. 1—*Absorption Spectra of Alkyl Halides*



1. 2, 3-DICHLOROPENTANE
2. 2, 4-DICHLOROPENTANE
3. 3-IODOBUTANE
4. 2-METHYL-1-IODOHEPTANE

caused the maximum to shift to 2000 Å and 2400 Å, respectively. This shift in position of the maximum shows the periodicity associated with the properties of the halogens, as was pointed out by Gillam and Morton[16] for comparative spectra of the free halogens. A study of five alkyl halides, by Levene, Rothen and Marker[17] showed that primary iodides absorb at slightly shorter wavelengths than do secondary

[16]Gillam, A. E., and Morton, R. A., *Proc. Roy. Soc.*, A. **124** (1929): 604.

[17]Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Phys.*, **4** (1936): 442.

iodides. Similar data for primary iodides was obtained by Fromherz.[18] In the five iodides he investigated, absorption was at about the same position in mono- and di-iodides with the exception of methylene iodide which showed the maximum at about 3100 Å. Hillmer and Paersch[19] in their work on alkyl iodides found that increase in carbon chain increased the strength of absorption, but that the position was apparently not a function of the alkyl group.[20] In the case of allyl bromide, Bandow[21] found, in a concentrated sulfuric acid solution, that the maximum was about 2700 Å. Investigation of unsaturated secondary halides by Levene and Rothen[22] showed that 3-chloro-1-heptene has considerably stronger absorption than has 4-chloro-1-pentene, and the authors concluded that the shorter the distance between the double bond and the functional group the stronger the absorption. The absorption of n-propyl-, butyl-, isopropyl-, and tertiary butyl-iodides was measured in atmospheres between 10^{-3} mm. to 10^{-4} mm. mercury or low temperatures between 150-180 Absolute and showed a number of fine bands.[23]

Simple Oxygen Derivatives

Alcohols: The saturated alcohols are generally non-absorbent in the near ultraviolet. Herzberg and Scheibe[15] investigated methanol by means of a vacuum spectrograph and found absorption between 1530-2000 Å with indications of an additional maximum at shorter wavelengths.

Allyl alcohol was studied by Bandow[21] in a concentrated sulfuric acid solution. Under the condition of his experiment

[18]Fromherz, H., *Z. Elektrochem.*, **43** (1937): 792.

[19]Hillmer, A., and Paersch, E., *Z. physik. Chem.*, A. **161** (1932): 46.

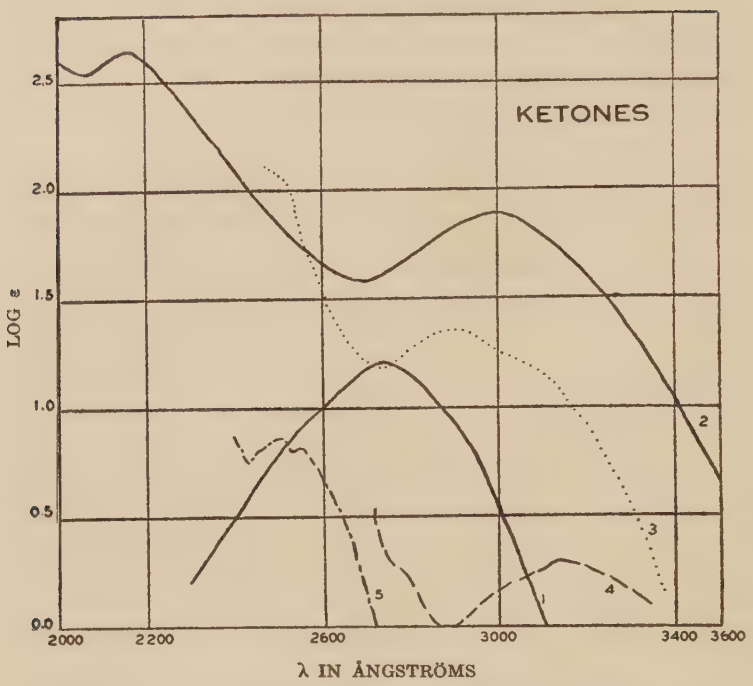
[20]Lowry, T. M., and Sass, J., *J. Chem. Soc.*, (1926): 622; Scheibe, G., *Ber.*, **58** (1925): 586; Sen-Gupta, P. K., *J. Univ. Bombay*, **5** (1936): 22.

[21]Bandow, F., *Biochem. Z.*, **298** (1938): 81.

[22]Levene, P. A., and Rothen, A., *J. Chem. Phys.*, **5** (1937): 980.

[23]Milazzo, G., *Z. physik. Chem.*, B. **33** (1936): 109.

FIG. 2—*Absorption Spectra of Ketones*



1. ACETONE 2. BROMOKETONE 3. 3-METHYLCYCLOHEXANONE
4. DIKETENE 5. β-BUTYRYLACETONE

he found a peak between 2750-2800 Å, which had a molecular extinction of 76,250. However, Bednarczyk and Marchlewski[24] found no selective absorption for allyl alcohol. Two unsaturated secondary carbinols, 1-pentene-4-ol and 1-heptene-3-ol were investigated by Levene and Rothen.[22] They found the alcohols to absorb less than the corresponding chlorides although in this case also, proximity of the double and the functional group enhances absorption.

Ethers: Simple ethers were studied by Scheibe and Grieneison.[7] Dimethyl ether has a weak band at 1870 Å and diethyl ether one at 1900 Å.

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[24]Bednarczyk, W., and Marchlewski, L., *Acad. polon. sci.*, A. (1937): 187.

Again employing concentrated sulfuric acid as a solvent, Bandow[21] found a weak maximum at 2900 Å for vinyl ethyl ether and two weak maxima for vinyl butyl ether, one at 3500 Å and the other at 2600 Å. In general Bandow concludes that the vinyl ethers are quite similar in absorption values to the allyl compounds investigated.

Carbonyl Derivatives

Ketones: Considerable data are available for acetone.[25] Ley and Arends[26] studied the carbonyl chromophores with vacuum spectrograph and found a peak between 1820 Å and 1910 Å, besides the maximum at 2700 Å. Mohler and Sorge[27] also investigated acetone in the short ultraviolet. By refined methods with a quartz spectrograph they located a maximum at about 2000-2150 Å for both acetone and bromoacetone. The curve continued upward but they were unable to reach the maximum pointed out by Ley and Arends because of limitations in their method. Rice,[28] Scheibe *et al.*,[29] and Wolf and Herold[30] studied the effect of solvent on the ketone position and strength. The conclusions to be drawn from the work are:

1. The aliphatic ketones with the exception of acetone and methyl ethyl ketone follow Beer's law.

2. Kundt's rule holds roughly.

3. When a ketone is dissolved in an ionizing type of solvent the absorption center moves toward the ultraviolet; in a non-polar solvent it is unaffected or moved toward the red.

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[25]Kuhn, W., and Gore, H. K., *Z. physik. Chem.*, B. 12 (1931): 389.

[26]Ley, H., and Arends, B., *ibid.*, B. 12 (1931): 132; Scheibe, G., and Linström, C. F., *ibid.*, B. 12 (1931): 387.

[27]Mohler, H., and Sorge, J., *Helv. chim. Acta*, 23 (1940): 100.

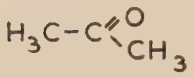
[28]Rice, F. O., *J. Am. Chem. Soc.*, 42 (1920): 727.

[29]Scheibe, G., *et al.*, *Ber.*, 60 (1927): 1407; *Z. Elektrochem.*, 34 (1928): 497.

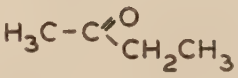
[30]Wolf, K. L., and Herold, W., *Z. physik. Chem.*, B. 5 (1929): 124.

4. The effect of the solvent is greater in lower molecular weight ketones.

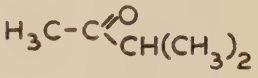
In the series:



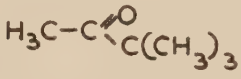
λ max. 2789 Å



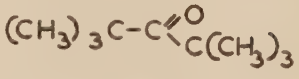
2797 Å



2845 Å



λ max. 2868 Å



2960 Å

Wolf[31] found that the shift of the carbonyl absorption band toward the red with addition of carbon chain length reached a boundary at 2814 Å for methyl ketones with a normal carbon chain. However, the substitution of a hydrogen by a methyl group on the adjacent carbon causes the absorption band to shift to longer wavelengths. The effect of analogous substitutions is very similar as is evident in the mass effect of

$$\Delta\lambda \text{ (acetone pinacolone)} = 79 \text{ Å and}$$

$$\Delta\lambda \text{ (pinacolone hexamethyl acetone)} = 92 \text{ Å}$$

A comparison of the effect of substitution of a methyl group for a hydrogen in the α-, β-, γ-positions is somewhat surprising. The effect of β-substitution is about twice that of α-substitution, while γ-substitution is barely noticeable. Furthermore Wolf, Briegleb and Stuart[32] concluded that the first sarbonyl absorption band in saturated aliphatic ketones is independent of the structure of the carbon residue.

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[31] Wolf, K. L., *ibid.*, B. 2 (1928): 39.

[32] Wolf, K. L., Briegleb, G., and Stuart, H. A., *ibid.*, B. 6 (1930): 168.

The effect of substituting a heavy atom such as bromine in acetone is to shift the entire spectrum about three hundred Ångstroms toward the red.

Saturated cyclic ketones have been studied by Herold[33], Donle and Volkert,[34] French and Naps,[35] Conrad-Billroth,[36] and Förster, Skrabal and Wagner.[37] In general the authors found smooth curves such as is shown in the figure; however, Förster, Skrabal and Wagner found that the broad absorption band generally given, could be resolved into a number of finer bands, whose position in wave number is a regular function of the number of members in the ring for cyclobutanone, cyclopentanone and cyclohexanone.

The absorption of β-butyryl acetone in 2,2,4-trimethylpentane was investigated by Calvin, Magel and Hurd[38] and in this compound the absorption is shifted to shorter wavelengths although there are two isolated chromophores besides the additional conjugated $-C=C-C=O$. Using the same solvent, diketene was found to have a band at 3130 Å, which was interpreted to indicate the presence of one pair of closely associated double bonds, one being $\rangle C=O$.[39] Förster *et al.*,[37] found ketene to show a band at 3250 Å which is considerably stronger than the absorption of the conjugated system of diketene. In the case of ketene, however, the absorption is due to the $\rangle C=C=O$ which acts as a single chromophore. The determinations of Scheibe[20] and Morton[40] show mesityl

[33]Herold, W., *ibid.*, B. 8 (1930): 265.

[34]Donle, H. L., Volkert, G., *ibid.*, B. 8 (1930): 60.

[35]French, H. S., and Naps, M., *J. Am. Chem. Soc.*, 58 (1936): 2303.

[36]Conrad-Billroth, H., *Z. physik. Chem.*, B. 14 (1931): 122.

[37]Förster, G., Skrabal, R., and Wagner, J., *Z. Elektrochem.*, 43 (1937): 290.

[38]Calvin, M., Magel, T., and Hurd, C. D., *J. Am. Chem. Soc.*, 63 (1941): 2174.

[39]Cooke, R. G., and Macbeth, A. K., *J. Chem. Soc.*, (1938): 1408.

[40]Morton, R. A., *J. Chem. Soc.*, (1926): 719.

oxide to have two well marked absorption bands which in alcohol are 3130 Å (e = 56) and 2380 Å (e = 14,000). Grossmann[41] pointed out that if a molecule has two chromophores the spectrum contains two bands and is shifted toward the red and intensified. The amount of the shift is dependent on the distance between the chromophores. In the case of mesityl oxide the 3100 Å band is that of the carbonyl while the 2300 Å band is that of the ethylene chromophore. The solvent affects the position of the maximum in this case, as was found by Rice for the saturated ketones. The band at 3130 Å, however, agrees well with the maximum for dehydroacetic acid. The position of the maximum for similar materials has led Aüsmuller, Fromherz and Strother[13] to conclude that although analogous loss of conjugation takes place in acetal, it is not followed by change to end absorption as might be expected. Hillmer and Paersch[42] found in comparing acetone, acetonylacetone, diacetyl and acetyl acetone that cumulative double bonds absorb at longer wavelengths than do the individual chromophores, but with not much increase in absorption strength. However, when the chromophores are so far separated as not to influence one another, the absorption strength is an additive effect of the absorption strengths of the individual chromophores.

Aldehydes: The saturated aldehydes show the maximum of the carbonyl group: formaldehyde in vapor state has two bands, one at 3350 Å and the other at 2530 Å.[43] Aldol shows approximately the same absorption as its parent aldehyde.

Again, as was found with the ketones, the conjugation of the carbonyl and ethylene chromophores causes a shift toward the red in acrolein, which shows a maximum at about 3200 Å.

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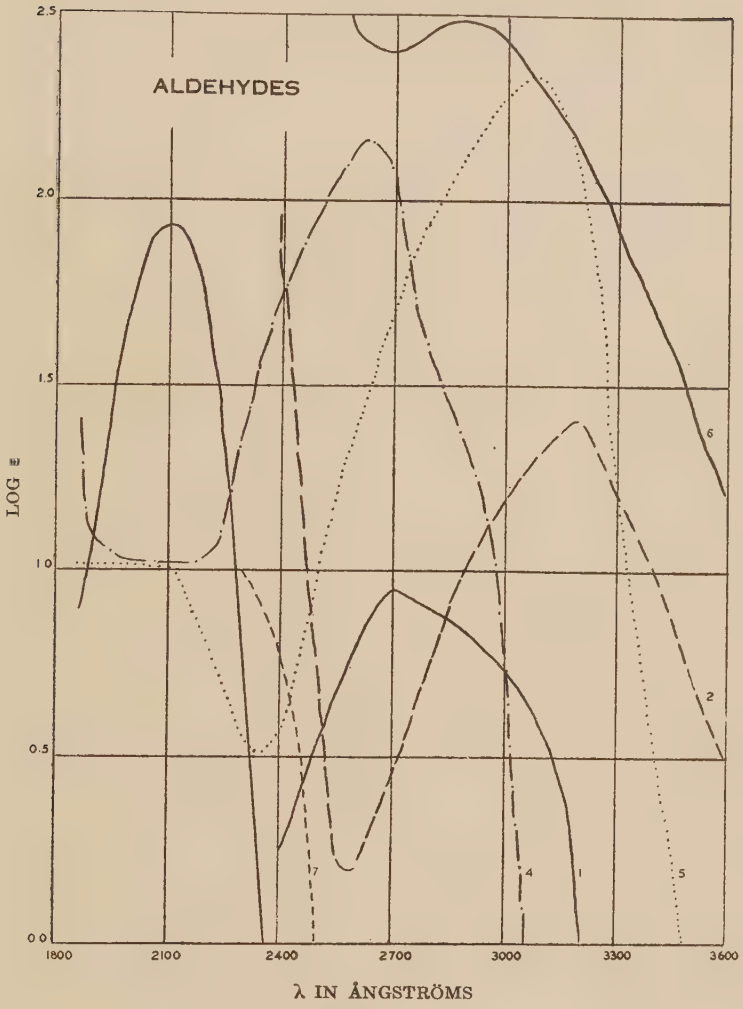
[41]Grossmann, O., *Z. physik. Chem.*, A. **109** (1924): 305.

[42]Hillmer, A., and Paersch, E., *ibid.*, A. **161** (1932): 58. *Cf.* Calvin, M., and Wood, C. L., *J. Am. Chem. Soc.*, **62** (1940): 3152.

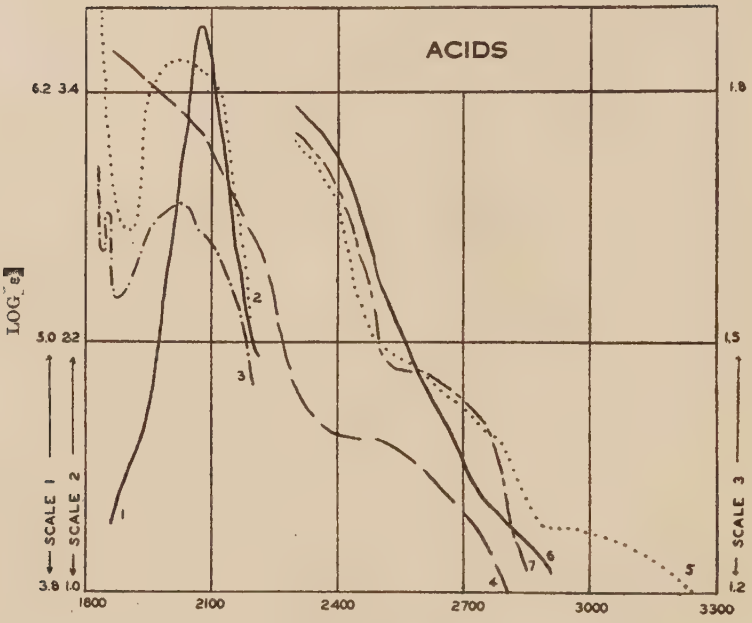
[43]Bredig, G., and Goldberger, A. V., *Z. physik. Chem.*, A. **110** (1924): 521.

FIG. 3—*Absorption Spectra of Aldehydes*



1. PROPIONALDEHYDE 2. ACROLEIN 3. CROTONALDEHYDE
4. SORBINALDEHYDE 5. OCTATRIENALDEHYDE 6. ALDOL
7. PARALDEHYDE

FIG. 4—*Absorption Spectra of Acids*



SCALE 2: 1. CROTONIC ACID 2. BUTRYIC ACID 3. ACETIC ACID
SCALE 1: 4. OXALIC ACID
SCALE 3: 5. MALEIC ACID 6. FUMARIC ACID 7. TETROLIC ACID

Such conjugation in crotonaldehyde, sorbinaldehyde and octatrienaldehyde (hexane solutions) absorbs at 2120 Å and shifts to 3060 Å with increase in molecular weight.[44] Thus in the cases of carbonyl-ethylene conjugation, the maximum is not as definitely localized in the aldehydes as it is in the ketones. Furthermore, Herold and Wolf[45] showed that the alcoholates and hydrates of simple aliphatic aldehydes are true valence combinations, which might account in part for the variation.

[44]Hausser, K. W., Kuhn, R., Smakula, A., and Hoffer, M., *ibid.*, B. **29** (1935): 371; Kuhn, R., and Hoffer, M., *Ber.*, **63** (1930): 2154.

[45]Herold, W., and Wolf, K. L., *Z. physik. Chem.*, B. **12** (1931): 165.

Acids: Ley and Arends[46] investigated the saturated carboxylic acids in the short wave ultraviolet and found that they generally were transparent out to about 2200 Å and that a maximum occurred between 2000-2200 Å. The maximum is shifted toward the red with increase in the carbon chain. Halogenated acids were studied by Kailen and Kunze[47] and no marked selectivity was found in the region investigated. There is no appreciable difference in the position of the maximum in butyric acid, as measured by Ley and Arends, and in the corresponding unsaturated acid (crotonic) measured by Hausser, Kuhn, Smakula and Hoffer,[44] which is not in agreement with the work of Aüsmuller, Fromherz and Strother,[18] who point out that carboxyl absorption is at 2050-2100 Å, and that conjugation of the carboxyl with the ethylene group shifts the maximum to 2400 Å. However, with increasing number of double bonds the main bands are shifted toward the visible and become increasingly higher in the crotonic acid series as is also the case of carbonyl-ethylene conjugation in aldehydes. Conjugation of two carboxyl groups causes the shift of the maximum to 2500 Å (oxalic acid), whereas two isolated carboxyl groups absorb in about the same position as a mono-carboxylic acid, with some increase in strength.

The differences in other physical properties in maleic and fumaric acids have led to the study of this pair of acids in several solvents by various workers. Bandow[21] sums up the knowledge by concluding that the absorption maxima are located in the short wave end of the ultraviolet and that there is very slight selectivity in the near ultraviolet. The difference in the spectra of *cis-* and *trans-*forms in any of the usual solvents is not marked.[48] Acetyl halides show about the same

[46]Ley, H., and Arends, B., *ibid.*, B. **17** (1932): 177; *ibid.*, B. **4** (1929): 234.

[47]Kailen, A., and Kunze, F., *Monatsh.*, **71** (1938): 373.

[48]Wassermann, A., and Smakula, A., *Z. physik. Chem.*, A. **155** (1931): 366; Ley, H., and Wingchen, H., *Ber.*, **67** (1934): 501.

absorption location as do the parent acids. However, the same periodicity in replacement of chlorine by bromine or iodine is noted in this type of compound as was found in the alkyl halides. Oxalyl bromide[49] shows no selectivity out as far as 2860 Å although the curve appears to be rising to a maximum toward 2000-2400 Å. The simple esters of carboxylic acids show only end absorption[50] as is also the case with the acid amides, which is not surprising in view of the work of Herold,[45] who found that substitution of a negative group causes a shift to shorter wavelengths.

Nitrogen Derivatives

Three important simple chromophores occur in the nitrogenous compounds, the nitro, the nitrile and the azo, besides the simple combinations with nitrogen. Ley and Arends[26] found only end absorption for diethyl amine. The tetraalkylammonium halides, investigated by Ali and Samuel,[51] in a series where the carbon number varies from one to five, show selective absorption when the carbon number is three or larger. In general, selective absorption is augmented with the heavier radicals, but in this series the chloride has stronger absorption than the bromide or iodide, which is not in agreement with the behavior of the alkyl halides. Since the principal maximum is at about the same wavelength (2700 Å) it seems reasonable to assume that it is independent of the halides and is characteristic of the hydrated R_4N ion. Körtum[52] found that tetraethylammonium nitrate had selective absorption at 3025 Å, which corresponds both in wavelength and strength with the value found, by Morton and Riding,[53] for the nitrate

[49]Tuttle, J., and Rollefson, G. K., *J. Am. Chem. Soc.*, 63 (1941): 1525.

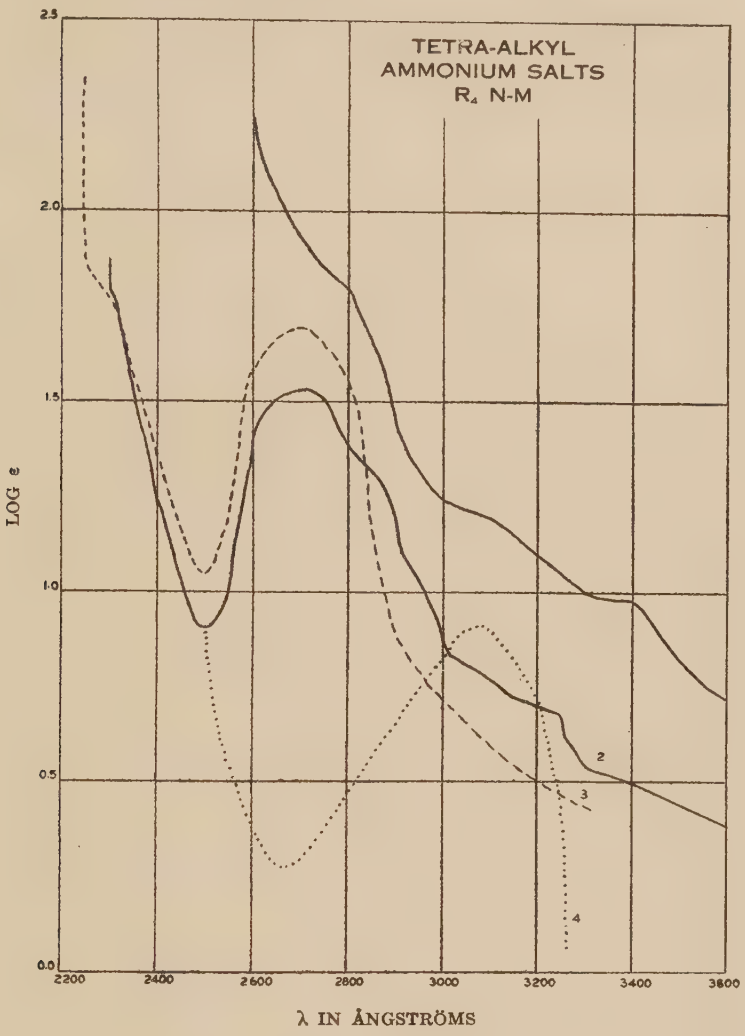
[50]Ley, H., and Hunecke, H., *Ber.*, 59 (1926): 510.

[51]Ali, S., and Samuel, R., *Proc. Indian Acad. Sci.* (III) A. (1936): 399; *Nature*, 137 (1936): 72.

[52]Körtum, G., *Ber.*, 71 (1938): 695.

[53]Morton, R. A., and Riding, R. W., *Proc. Roy. Soc.*, A. 113 (1927): 717.

FIG. 5—*Absorption Spectra of Tetra-Alkyl Ammonium Salts*



1. $(C_3H_7)_4NI$ 2. $(C_3H_7)_4NBr$ 3. $(C_3H_7)_4NCl$ 4. $(C_2H_5)_4NNO_3$

group. These latter authors report that the simple alkyl nitrates do not exhibit selective absorption and postulate the absorption in the region of 3000 Å to be due to the presence of the undissociated molecules. If this be true, the hydrated

R_4N ion is likewise present in the case of tetraalkylammonium nitrate, and its absorption is covered by that of the nitrate ion. The absorption band was found in the same place for tetrapropylammonium nitrate by Halban and Eisenband[54] and it was concluded that shifts to the ultraviolet indicate nondissociation or association of ions whereas shifts toward the red show complex formation or association of molecules.

Nitro Compounds: Nitromethane shows a shift in the position of the maximum from 2400 Å in basic solution to 2800 Å in acid. The effect of increasing the number of nitro groups successively up to four is to shift the maximum to longer wavelengths up to three groups and then back slightly for four.[55] In the case of nitrosyl chloride[56] there are two maxima, at 1970 Å and 3350 Å, respectively. Körtum[57] found a weak band for ethyl nitrate at about 2850 Å. From these data it is indicated that the $-N=O$ group absorbs in the region of 2800-3500 Å.[58]

The Nitriles: The ethylenic nitriles were investigated by Castille and Ruppol,[59] who found that the *trans*-form absorbs more than the *cis*-form in the crotonic series. In this series absorption varies with the molecular weight, contrary to observations in other homologous series containing a normal chain with only one active group.[60] However, it is difficult to fix the maximum of the absorption bands because they are considerably spread out. The nitriles of dicarboxylic acids again show only a general absorption.

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[54]Halban, H., and Eisenband, J., *Z. physik. Chem.*, A. **146** (1930): 294.

[55]Körtum, G., *ibid.*, B. **43** (1939): 271.

[56]Goodeve, C. F. and Katz, S., *Proc. Roy. Soc.*, A. **172** (1939): 432.

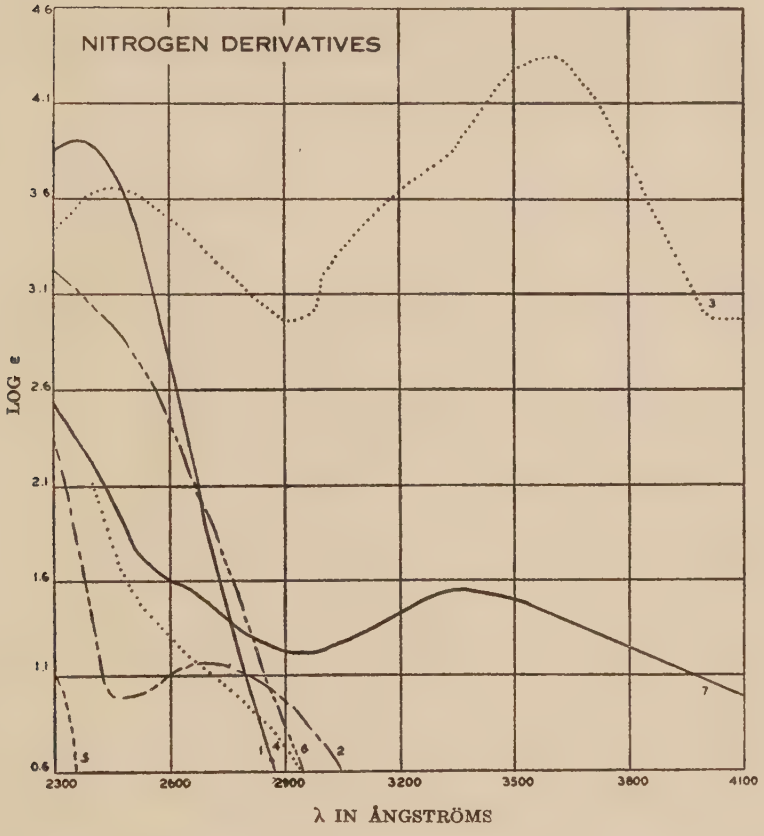
[57]Körtum, G., *Z. physik. Chem.*, B. **43** (1939): 418.

[58]Kuhn, W., and Biller, H., *ibid.*, B. **29** (1935): 1.

[59]Castille, A., and Ruppol, E., *Bull. soc. chim. Belg.*, **44** (1935): 351.

[60]Ramart-Lucas, P., Biquard, D., and Grunfeld, M., *Compt. rend.*, **190** (1930): 1196; Grunfeld, M., *Ann. chim.*, **20** (1933): 312.

FIG. 6—*Absorption Spectra of Nitrogen Derivatives*



1. CH_3NO_2 in 0.01 N. NaOH
2. CH_3NO_2 in 0.01 N. HCl
3. $CH_2(NO_2)_2$
4. $C_2H_5NO_2$ in DIOXANE
5. $(CH_3)_2NH$
6. dl-α-BROMONITRILE
7. NITROSYLCHLORIDE

Azo Derivatives

In a comparison of the spectra of azo-methane and hexafluoromethane, Ruff and Willenberg[61] observed that the replacement of hydrogen by fluorine caused a marked decrease in the strength of the maximum.

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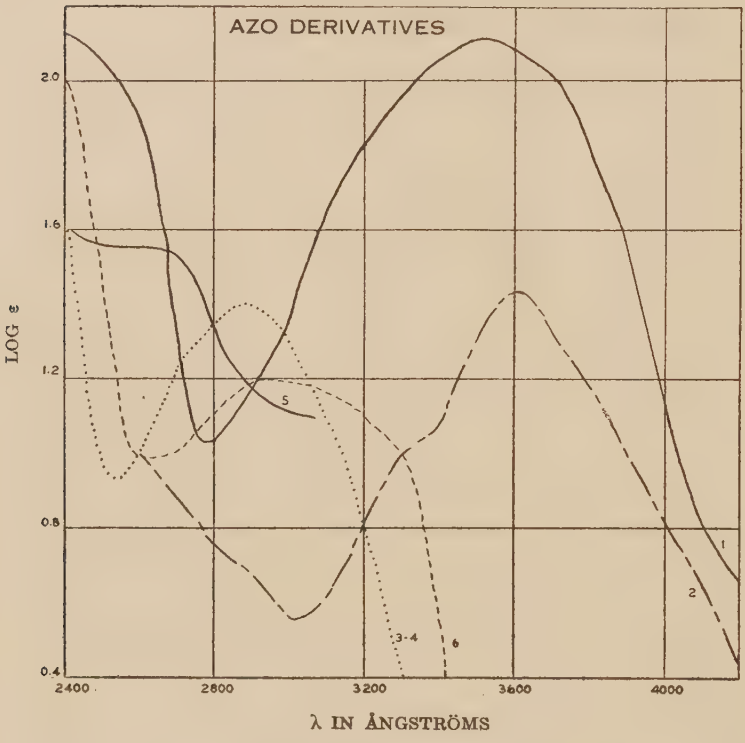
[61]Ruff, O., and Willenberg, W., *Ber.*, **73** (1940): 724.

FIG. 7—*Absorption Spectra of Azo Derivatives*



1. $H_3C-N=N-CH_3$ 2. $F_3C-N=N-CF_3$ 3. $HC(CH_3)(C_6H_{13})N$ (N=N)

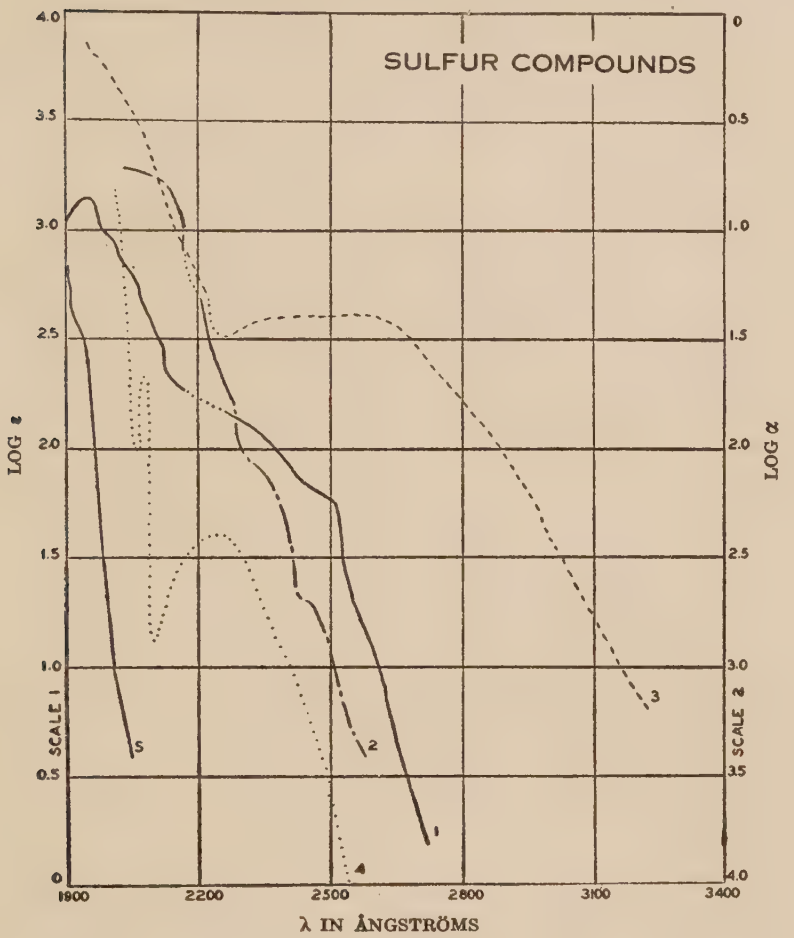
4. $HC(CH_3)(C_2H_5)CH_2CH_2N$ (N=N) 5. $CH_3CH-N_3(COOCH_3)$ 6. HN_3

The simplest of the tri-azo compounds, hydrazoic acid,[62] shows a slight selectivity in the region of 2800 Å. Levene and Rothen[63] investigated a number of alkyl azides and found them all to have characteristic absorption at 2880 Å, the intensity of which is independent of the alkyl group and is uninfluenced

[62]Garner, W. E., and Maggs, J., *Proc. Roy. Soc.*, A. **122** (1939): 299.

[63]Levene, P. A., and Rothen, A., *J. Chem. Phys.*, **5** (1937): 985.

FIG. 8—*Absorption Spectra of Sulfur Compounds*

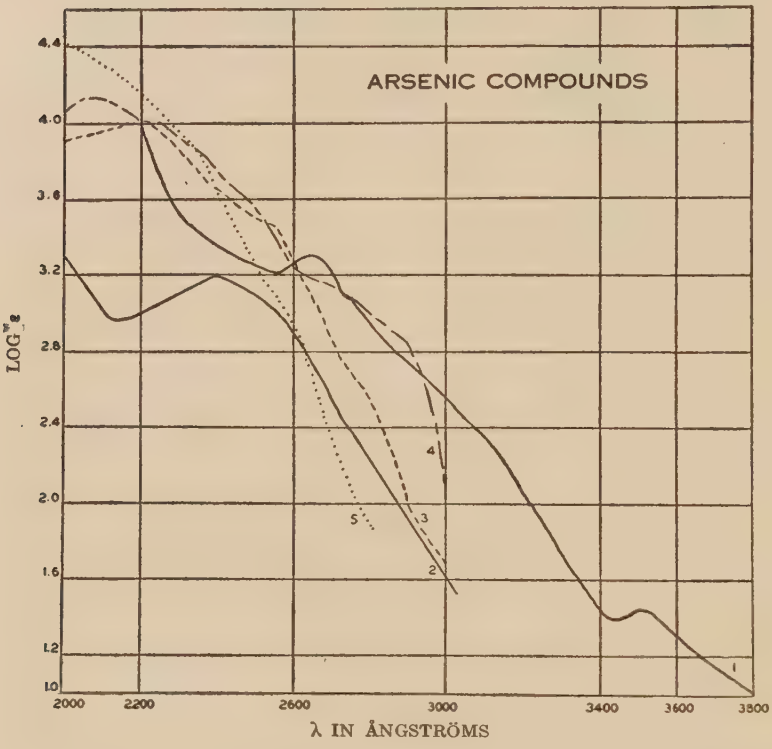


1. C_2H_5SH 2. $(C_2H_5)_2S$ 3. $C_2H_5SSC_2H_5$ 4. $O=C=S$ 5. $S(C_2H_5)_3Cl$

by the carbonyl or phenyl groups. Azido alcohol, azidomethyl propionate and azido propionyl dimethylamide[64] all show the characteristic band at 2800 Å, which may be ascribed to

[64]Kuhn, W., and Braun, E., *Z. physik. Chem.*, B. 8 (1930): 281; *ibid.*, B. 8 (1930): 445.

FIG. 9—*Absorption Spectra of Arsenic Derivatives*



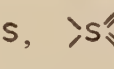
1. $(C_6H_5)_2AsCl$ 2. $C_2H_5AsCl_2$ 3. $ClCH=CHAsCl_2$ 4. $(ClCH=CH)_2AsCl$
5. $(ClCH=CH)_3As$

the N_3 group. When the N_3 group occurs in more complex compounds, however, as for example, N_3CS_2H (Na) the band is shifted to longer wavelengths.[65]

Sulfur Derivatives

Sulfur dioxide absorbs at 2800 Å and this band is shifted to longer wavelengths in Na_2SO_3 and $Na_2S_2O_4$. Sodium methyl sulfate has an indistinct band at 2800-3000 Å, while

[65]Hantzsch, A., and Bucerius, B., *Ber.*, **59** (1926): 793.

dimethyl sulfate[66] absorbs with a decided maximum at 2800 Å. Ley and Arends[67] found a maximum for ethyl mercaptan at 1935 Å. Diethyl sulfide was measured out to 2000 Å, at which position the maximum was not located, although the sharp rise would seem to indicate a maximum at about 1900 Å. Gaseous carbonyl sulfide has a maximum at 2250 Å and another at 2080 Å. In general it seems that compounds of the type R-S-H and R-S-R absorb at about 1900 Å and so resemble the corresponding alcohols and ethers. Disulfides show a shift in the position of the maximum to 2400-2600 Å. Sulfur groups showing unsaturation absorb in the near ultraviolet:  , give absorption maxima between 2700-3000 Å.

Arsenic Derivatives

Mohler and Sorge[68] investigating a series of seven arsenicals found in comparing saturated and unsaturated carbon substituents, that unsaturation causes a shift toward shorter wavelengths. Increase in the number of vinyl groups causes further shift to shorter wavelengths, which decreases with each subsequent vinyl group. From the data thus far presented no assignment of position of maxima can be made.

Summary

The present study of the available literature on the absorption spectra of organic compounds permits the following generalizations:

1. Saturated hydrocarbons show no selective absorption in the quartz ultraviolet.

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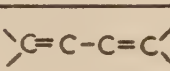
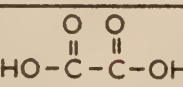
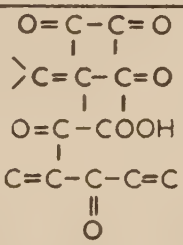
[66]Lorenz, L., and Samuel, R., *Z. physik. Chem.*, B. 17 (1932): 177; Karin, S. M., and Samuel, R., *Proc. Indian Acad. Sci.*, 1 (1934): 398.

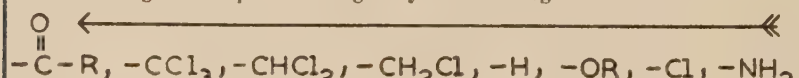
[67]Ley, H., and Arends, B., *Z. physik. Chem.*, B. 15 (1931): 311.

[68]Mohler, H., and Sorge, J., *Helv. chim. Acta*, 23 (1940): 104.

SUMMARY

Group	Absorption in Å	Conjugation	Absorption in Å
C–C	<1530		
C=C	1850		2200
	position not located: evidently below 2000		
	2080		
–CO–OH	2080		2500
	2700		2860 3140 3310 3400
–N=O	3020		3660
–N=N–	3500		2880
S=O	2700-3000		

Increasing chromophore strength by substituting:



2. The simple ethylene linkage absorbs at the limits of the quartz ultraviolet. On conjugation the absorption center is shifted toward the red.

3. The data reviewed were not obtained with equipment designed to extend beyond 2000 Å and the center of the >C=N absorption has not been located.

4. >C=S absorbs at 2080 Å.

5. The carboxyl group absorbs at 2080 Å but on conjugation there is considerable shift toward the red.

6. The carbonyl group absorbs at 2700 Å and conjugation of carbonyls causes a slight shift toward the red. Conjugation with the ethylene linkage causes a more pronounced shift. Conjugation with carboxyl shows additional shift toward the red.

7. The nitroso group absorbs at 3020 Å and the nitro group at 3660 Å.

8. The azo group absorbs at 3500 Å, whereas the triazo group absorbs further to the violet, at 2880 Å.

9. Sulfoxide absorbs between 2700-3000 Å.

10. In general the effect of substitution next to a chromophore is to cause a shift to longer wavelength. Maximum shift to the red is caused by $-C\begin{matrix}O\\ \diagdown R\end{matrix}$ while hydrogen has very little effect and negative groups cause shift toward the violet so that carbonyl derivatives, for example, with a negative group are colorless and those with two negative groups do not absorb in the quartz ultraviolet.

The authors wish to acknowledge the assistance of Dr. Elton S. Cook of the Institutum Divi Thomae, Dr. John R. Loofbourow of the Massachusetts Institute of Technology, and Sister Mary Jane Hart, O.P., of Barry College, in accumulating and correlating data. They also extend thanks to Miss Mary Baldwin for clerical assistance and to Miss Lilia Dominguez for construction of the graphs.

LITERATURE CITED

Ali, S., and Samuel, R., *Proc. Indian Acad. Sci.* (III) A. (1936): 399; *Nature*, **137** (1936): 72.

Ausmüller, W., Fromherz, H., and Strother, C., *Z. physik. Chem.*, B. **37** (1937): 30.

Bandow, F., *Biochem. Z.*, **298** (1938): 81.

Bednarczyk, W., and Marchlewski, L., *Acad. polon. sci.*, A. (1937): 187.

Booker, H., Evans, L. K., and Gillam, A. E., *J. Chem. Soc.*, (1940): 1453.

Bradley, T. E., and Richardson, D., *Ind. Eng. Chem.*, **32** (1940): 963.

Bredig, G., and Goldberger, A. V., *Z. physik. Chem.*, A. **110** (1924): 521.

Brode, W. R., Chemical Spectroscopy, Wiley and Sons, 1939, New York, pp. 124-140.

Calvin, M., Magel, T., and Hurd, C. D., *J. Am. Chem. Soc.*, **63** (1941): 2174.

Calvin, M., and Wood, C. L., *J. Am. Chem. Soc.*, **62** (1940): 3152.

Carr, E. P., and Stücklen, H., *Z. physik. Chem.*, B. **25** (1934): 57.

Carr, E. P., and Walker, M. K., *J. Chem. Phys.*, **4** (1936): 751.

Castille, A., and Ruppol, E., *Bull. soc. chim. Belg.*, **44** (1935): 351.

Conrad-Billroth, H., *Z. physik. Chem.*, B. **14** (1931): 122.

Cooke, R. G., and Macbeth, A. K., *J. Chem. Soc.*, (1938): 1408.

Dilthey, W., *Ber.*, **53** (1920): 261.

Donle, H. L., Volkert, G., *Z. physik. Chem.*, B. 8 (1930): 60.

Dutt, S., *J. Chem. Soc.*, (1926): 1171; *J. Indian Chem. Soc.*, **4** (1927): 99.

Förster, G., Skrabal, R., and Wagner, J., *Z. Elektrochem.*, **43** (1937): 290.

French, H. S., and Naps, M., *J. Am. Chem. Soc.*, **58** (1936): 2303.

Fromherz, H., *Z. Elektrochem.*, **43** (1937): 792.

Garner, W. E., and Maggs, J., *Proc. Roy. Soc.*, A. **122** (1939): 299.

Gillam, A. E., and Morton, R. A., *Proc. Roy. Soc.*, A. **124** (1929): 604.

Goodeve, C. F., and Katz, S., *Proc. Roy. Soc.*, A. **172** (1939): 432.

Grossmann, O., *Z. physik. Chem.*, A. **109** (1924): 305.

Grunfeld, M., *Ann. chim.*, **20** (1933): 312.

Halban, H., and Eisenband, J., *Z. physik. Chem.*, A. **146** (1930): 294.

Hantzsch, A., and Bucerius, B., *Ber.*, **59** (1926): 793.

Hausser, K., Kuhn, R., and Seitz, G., *Z. physik. Chem.*, B. **29** (1935): 391.

Hausser, K., Kuhn, R., and Smakula, A., *ibid.*, B. **29** (1935): 384.

Hausser, K. W., Kuhn, R., Smakula, A., and Hoffer, M., *ibid.*, B. **29** (1935): 371.

Herold, W., and Wolf, K. L., *ibid.*, B. **12** (1931): 165.

Herold, W., *ibid.*, B. **8** (1930): 265.

Herzberg, G., and Scheibe, G., *ibid.*, B. (1940): 390.

Hillmer, A., and Paersch, E., *ibid.*, A. **161** (1932): 46, 58.

Kailen, A., and Kunze, F., *Monatsh.*, **71** (1938): 373.

Karin, S. M., and Samuel, R., *Proc. Indian Acad. Sci.*, **1** (1934): 398.

Körtum, G., *Ber.*, **71** (1938): 695; *Z. physik. Chem.*, B. **43** (1939): 271, 418.

Kuhn, R., and Hoffer, M., *Ber.*, **63** (1930): 2154.

Kuhn, W., and Biller, H., *ibid.*, B. **29** (1935): 1.

Kuhn, W., and Braun, E., *ibid.*, B. **8** (1930): 281, 445.

Kuhn, W., and Gore, H. K., *ibid.*, B. **12** (1931): 389.

Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Phys.*, **4** (1936): 442.

Levene, P. A., and Rothen, A., *ibid.*, **5** (1937): 980, 985.

Lewis, G. N., and Calvin, M., *Chem. Rev.*, **25** (1939): 273.

Ley, H., and Arends, B., *Z. physik. Chem.*, B. **4** (1929): 234; B. **12** (1931): 132; B. **15** (1931): 311; B. **17** (1932): 177.

Ley, H., and Hunecke, H., *Ber.*, **59** (1926): 510.

Ley, H., and Wingschen, H., *Ber.*, **67** (1934): 501.

Lorenz, L., and Samuel, R., *Z. physik. Chem.*, B. **17** (1932): 177.

Lowry, T. M., and Sass, J., *J. Chem. Soc.*, (1926): 622.

Marvel, C. S., Sample, J. H., and Ray, M. F., *J. Am. Chem. Soc.*, **61** (1939): 3241.

Milazzo, G., *Z. physik. Chem.*, B. **33** (1936): 109.

Miller, E. S., Quantitative Biological Spectroscopy, 2nd edition, 1940, Burgess Publishing Company, Minneapolis, Minnesota.

Mohler, H., and Sorge, J., *Helv. chim. Acta*, **23** (1940): 100, 104.

Morton, R. A., *J. Chem. Soc.*, (1926): 719.

Morton, R. A., and Riding, R. W., *Proc. Roy. Soc.*, A. **113** (1927): 717.

Ramart-Lucas, P., Biquard, D., and Grunfeld, M., *Compt. rend.*, **190** (1930): 1196.

Rice, F. O., *J. Am. Chem. Soc.*, **42** (1920): 727.

Ruff, O., and Willenberg, W., *Ber.*, **73** (1940): 724.

Scheibe, G., *Ber.*, **58** (1925): 586; **59** (1926): 1333.

Scheibe, G., *et al.*, *Ber.*, **60** (1927): 1407; *Z. Elektrochem.*, **34** (1928): 497.

Scheibe, G., and Grieneison, H., *Z. physik. Chem.*, B. **25** (1934): 52.

Scheibe, G., and Linström, C. F., *ibid.*, B. **12** (1931): 387.

Sen-Gupta, P. K., *J. Univ., Bombay*, **5** (1936): 22.

Tuttle, J., and Rollefson, G. K., *J. Am. Chem. Soc.*, **63** (1941): 1525.

Wassermann, A., and Smakula, A., *Z. physik. Chem.*, A. **155** (1931): 366.

Wolf, K. L., *Z. physik. Chem.*, B. **2** (1928): 39.

Wolf, K. L., Briegleb, G., and Stuart, H. A., *ibid.*, B. **6** (1930): 163.

Wolf, K. L., and Herold, W., *Z. physik. Chem.*, B. **5** (1929): 124.

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October 25, 1941

GROWTH FACTORS FOR BACTERIA*

SISTER MARY PETRONELLA SCHROEDER, C.PP.S., AND
REVEREND CHARLES H. HOLLENCAMP

Cook and other workers in these laboratories[1] described, in a preliminary report, substances obtained from yeast which affected the growth of bacteria. The present research has as its object an extension of the study of these yeast fractions on the growth of bacteria and an attempt to isolate these growth-promoting substances. In addition, a study of the morphological changes effected in the growth of bacteria by various yeast fractions is presented. Departure from the characteristic morphology has frequently been referred to in the literature. Wilson[2] found that when Bacillus coli, Bacillus typhosus, Bacillus enteriditis, the Friedlander bacillus, and the plague bacillus were cultivated on media containing urine, a great diversity of distorted forms and filamentous elements were noted. It has been shown that the addition of magnesium chloride[3] and dyes[4] to media leads to the production of atypical forms of rod-shaped organisms. Reed and Orr[5] attribute such morphological changes to the influence of hydrogen-ion concentration in the media. According to Henrici,[6] who has made a special study of atypical forms in Bacillus coli, morphological variations occur in the lag period of bacterial growth. He correlates the phenomenon with autolysis of dead cells. Branch-

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[1]Cook, E. S., Kreke, C. W., Giersch, Sr. M. C., and Schroeder, Sr. M. P., *Science*, **93** (1941): 616.

[2]Wilson, W. J., *J. Path. Bact.*, **11** (1906): 394.

[3]Hata, S., *Centr. Bakt. Parasitenk.*, **46** (1908): 289.

[4]Vay, F., *Centr. Bakt. Parasitenk.*, **55** (1910): 193.

[5]Reed, G., and Orr, J. H., *J. Bact.*, **8** (1923): 103.

[6]Henrici, A. T., *J. Infectious Diseases*, **38** (1926): 54; **39** (1926): 429.

ing filamentous forms have also been described by Gardner,[7] who believes that probably many pathogenic bacilli grow by three-point branching from Y-shaped forms.

EXPERIMENTAL

The fractions of yeast used in these experiments were reduced to dryness at 52° C., dissolved in water, and the pH adjusted to 7.2. In this form they were incorporated into Difco nutrient agar in concentrations varying from 0.5 to 10 per cent. After being autoclaved the nutrient agar containing these fractions was poured into sterile petri plates, 10 ml. being used for the larger plates and 2 ml. for the smaller ones. The plates were inverted and left to stand until the superfluous moisture disappeared from the surface of the hardened medium. Then a very small amount of a stock culture (18 to 24 hours old) of E. coli, grown on nutrient agar in a large petri plate, was transferred to each of the above mentioned petri plates by the following method: The end of a platinum wire was formed into an isosceles triangle with the vertex angle about 30 degrees and the shaft of the wire as the base. The apex of this triangle formed the charging point of the loop. It was touched lightly on the latest growth of a stock culture of E. coli and then was brought lightly into contact with the surface of the nutrient agar on the plates containing the varying concentrations of the fractions. When large petri plates were used, five colonies were transferred to each plate. With small petri plates, only one colony was transferred to the center of each plate.

In some cases the fractions were also incorporated into Difco nutrient broth in proportions varying from 0.5 to 10 per cent, the resulting mixture in each instance being 5 ml. After autoclaving and subsequent cooling, this liquid medium was

[7]Gardner, A. D., *J. Path. Bact.*, 28 (1925): 189.

inoculated by the transfer of one loopful of an 18 to 24-hour nutrient broth culture. Both the solid and liquid cultures were incubated for 4 days at 37.5° C.

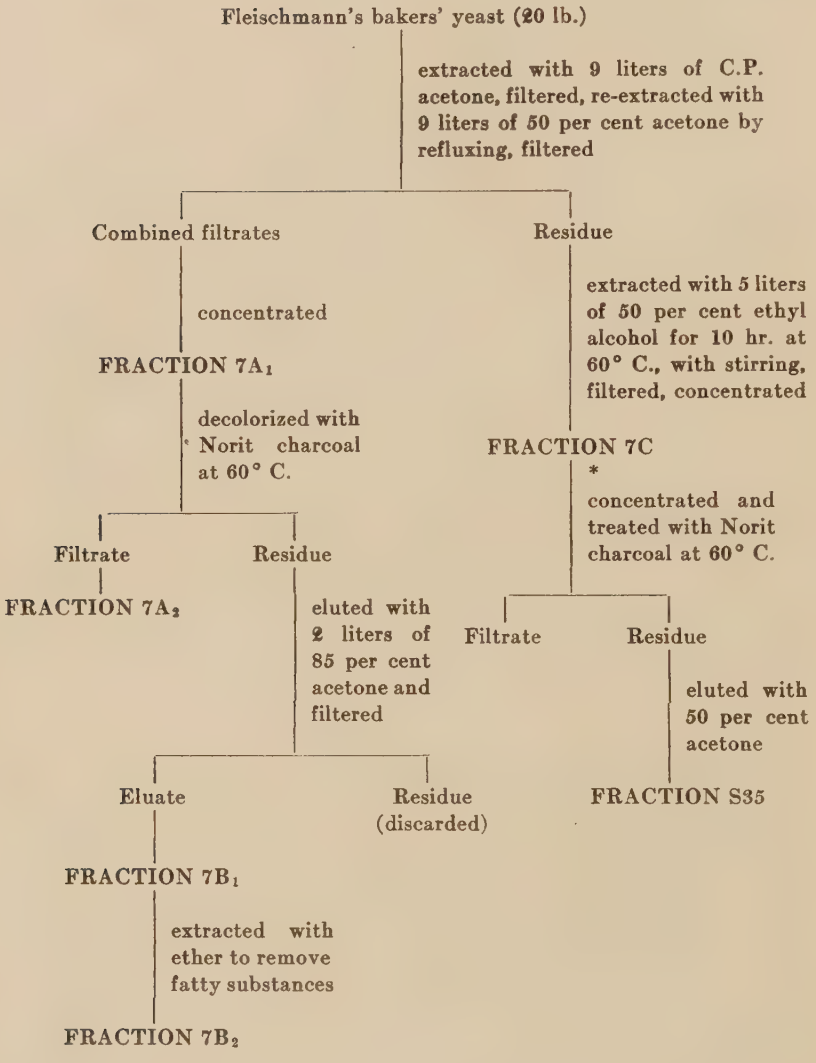
Quantitative measurement of the spread and growth of the colonies was attempted by tracing the areas of the colonies through the inverted petri plates on onion skin paper and measuring these areas with a planimeter. Five planimeter readings were made of the area of each colony. The average of these readings was taken as the area of the colony. When large petri plates were used an average was made of the five colony areas. Since the areas alone do not adequately express the amount of growth but only the spreading effect on colony size, photographs of the 4-day cultures were made to show the comparative densities of the cultures and other characteristics of the colonies. Plates I and II show typical colonies obtained. In the experiments in which broth cultures were used to parallel the experiments on solid media, quantitative measurements were made by sedimentation of the bacterial growth in Hopkins centrifuge tubes at 1400 revolutions per minute for one-half hour.

DISCUSSION

Figure 1 summarizes the preparation of Fractions $7A_1$, $7A_2$, $7B_1$, $7B_2$, and 7C. The first four were acetone extracts of Fleischmann's bakers' yeast. 7C was an alcoholic extract of the residue obtained after the first acetone extraction of the yeast. Three other fractions, 8C, 8B, and S35, were derived somewhat differently. Their preparation will be mentioned as they are discussed. In addition to these, two other alcoholic fractions were investigated: one prepared like 7C, the other by a method to be described later.

Plate I presents a picture of the cultural characters of the colonies resulting from the incorporation of the various fractions derived according to the procedure outlined in Fig. 1.

Fig. 1


Fleischmann's bakers' yeast (20 lb.)
extracted with 9 liters of C.P. acetone, filtered, re-extracted with 9 liters of 50 per cent acetone by refluxing, filtered
Combined filtrates
Residue
concentrated
FRACTION 7A1
extracted with 5 liters of 50 per cent ethyl alcohol for 10 hr. at 60° C., with stirring, filtered, concentrated
decolorized with Norit charcoal at 60° C.
FRACTION 7C
*
concentrated and treated with Norit charcoal at 60° C.
Filtrate
Residue
FRACTION 7A2
eluted with 2 liters of 85 per cent acetone and filtered
Filtrate
Residue
eluted with 50 per cent acetone
Eluate
Residue (discarded)
FRACTION S35
FRACTION 7B1
extracted with ether to remove fatty substances
FRACTION 7B2

*The portion following the asterisk shows the derivation of Fraction S35, not from extract 7C but from another extract corresponding to it and derived from a different batch of Fleischmann's yeast.

PLATE I

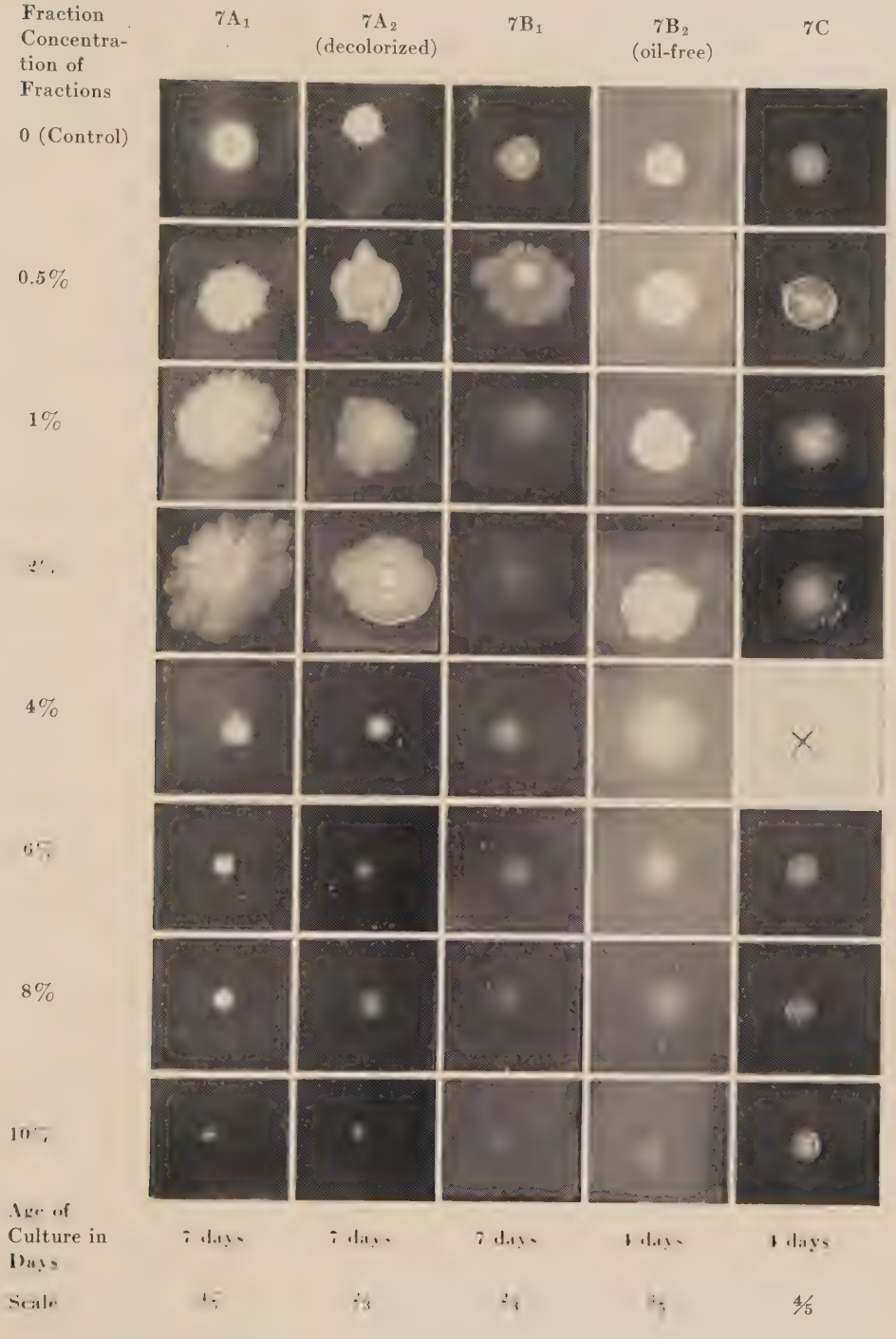

Fraction Concentration of Fractions
7A1
7A2 (decolorized)
7B1
7B2 (oil-free)
7C
0 (Control)
0.5%
1%
2%
4%
6%
8%
10%
Age of Culture in Days
7 days
7 days
7 days
4 days
4 days
Scale
4/5
4/5
4/5
4/5
4/5

PLATE II

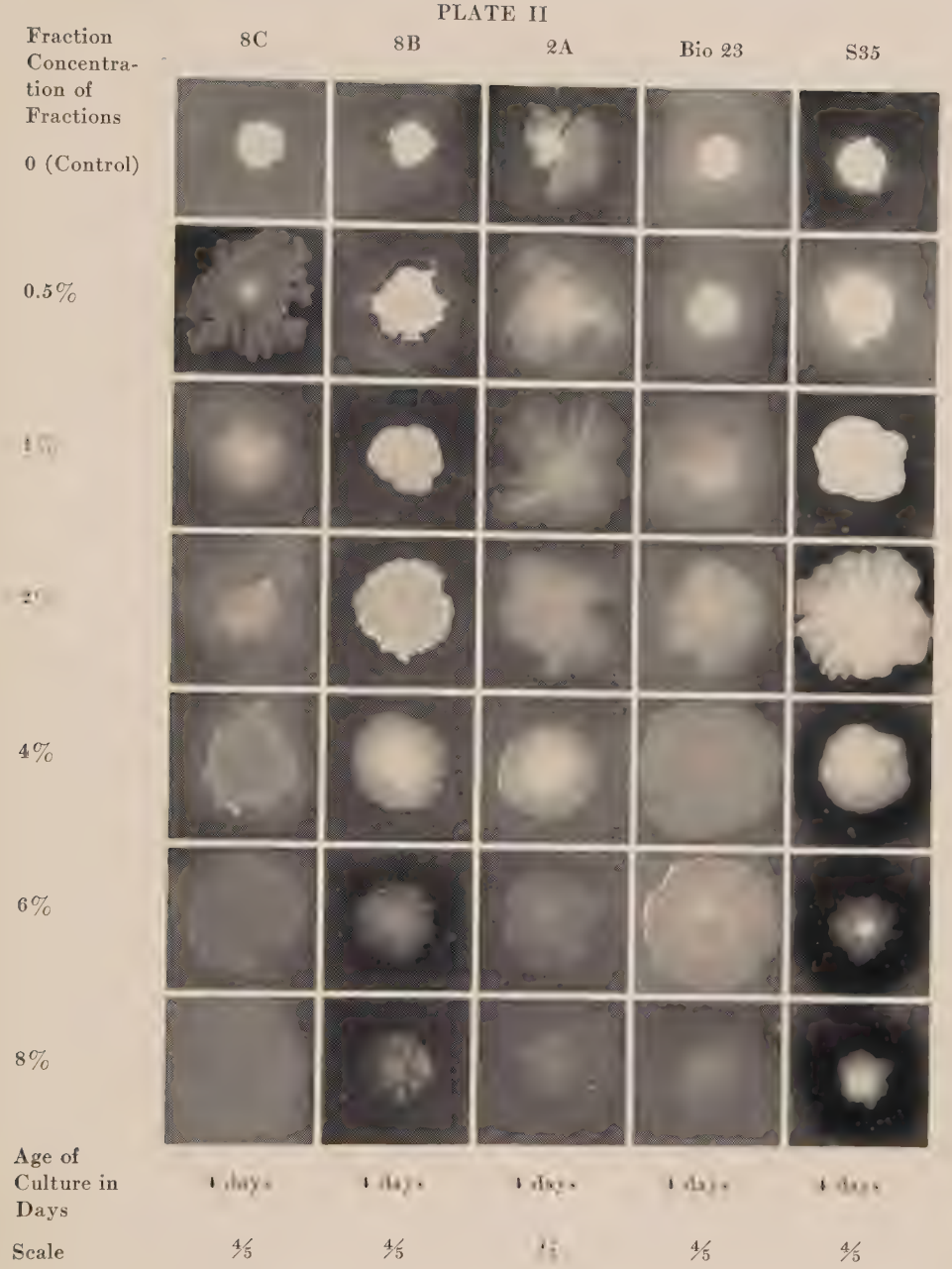
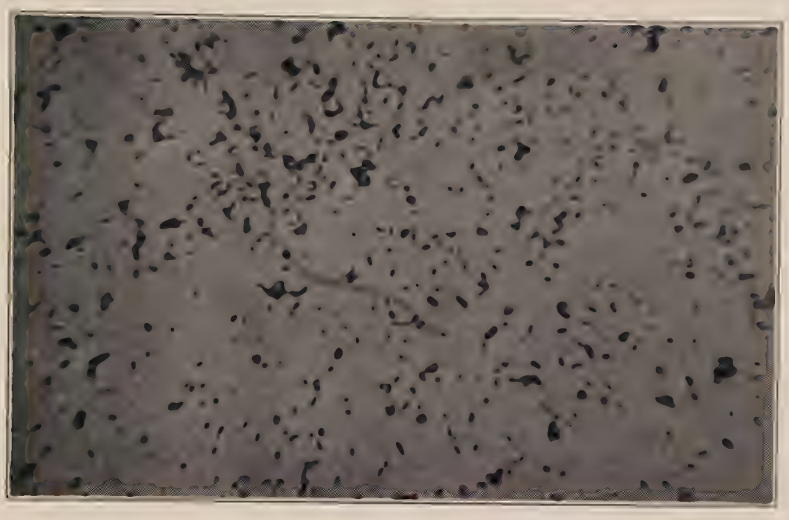
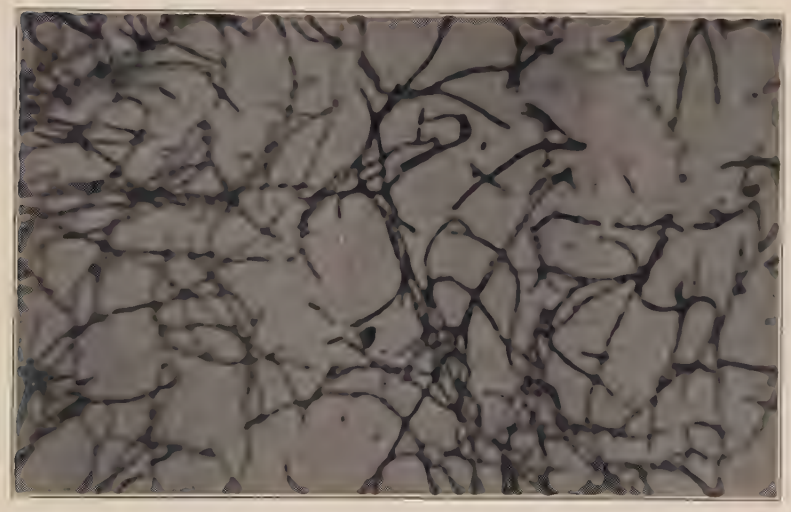

Fraction Concentration of Fractions
8C
8B
2A
Bio 23
S35
0 (Control)
0.5%
1%
2%
4%
6%
8%
Age of Culture in Days
4 days
4 days
4 days
4 days
4 days
Scale
4/5
4/5
4/5
4/5

PLATE III



Escherichia Coli from Control Culture (X 1440)



Escherichia Coli in Filamentous Form (X 1440)

When studying this plate it should be kept in mind that the colonies pictured in columns $7A_1$, $7A_2$, and $7B_1$ were 7 days old, while those in $7B_2$ and 7C were only 4 days old.

The modification of the nutrient agar by incorporation of Fraction $7A_1$ in concentrations of 0.5 to 2 per cent caused a notable increase of area. In concentrations higher than these a marked inhibition in growth was noted. The higher the concentration, the smaller the colonies became, until they were scarcely visible on the plates containing 10 per cent of this fraction. The growth on the outer boundary of the colonies grown on the media containing the four highest concentrations was characterized as very sparce and granular. Microscopically, too, the characteristics of E. coli had been altered. Briefly, the change consisted in the conversion of normal short bacilli (1 to 3 microns) into organisms of greater length (6 to 8 microns) in the media containing 4 per cent of Fraction $7A_1$ and finally, in the media containing 6, 8 and 10 per cent of Fraction $7A_1$ into forms which presented themselves almost entirely as long filaments or threads. These filaments were often 50 and more times the length of the ordinary bacillus and showed no sign of segmentation. They frequently presented the appearance of true branching. The photomicrographs in Plate III show E. coli as seen in Wright-stained smears from the control plates and from plates containing 6 per cent of this fraction. The organisms on plates containing 8 and 10 per cent were similar in appearance to those on plates containing 6 per cent.

The fact that yeast fractions both stimulated and depressed the growth of E. coli suggested the presence of two antagonistic factors, the one growth-promoting, the other growth-depressing. Accordingly, Fraction $7A_1$ was adsorbed on charcoal to see whether the two factors might be separated. Both the adsorbed Fraction $7B_1$ and the non-adsorbed Fraction $7A_2$ were embodied in nutrient agar and seeded with E. coli, as previously

described. On comparing the cultural characters of the colonies produced on these media (Plate I, Columns $7A_2$ and $7B_1$) we note that Fraction $7A_2$ seems to contain the factor which, in concentrations up to 2 per cent, favors growth. The charcoal-adsorbed fraction also contains a growth-promoting factor but seemingly to a lesser degree. The spreading effect of the $7B_1$ colonies may be attributed in part to the presence in Fraction $7B_1$ of a fatty substance which reduced the surface tension of the media and so caused the colonies to extend over comparatively large surfaces. Their density became so reduced that they were nearly transparent and so were rather difficult to photograph. The growth obtained with 1 per cent and 2 per cent of this fraction was spread over areas even greater than those shown on the plate. In this case it is obvious that measurement of colony area is not an adequate criterion of bacterial growth. Microscopic examination of the organisms revealed characters as described for Fraction $7A_1$.

The purification process was carried a step further. The fatty substance was removed from Fraction $7B_1$ and the resulting oil-free fraction called Fraction $7B_2$. This fraction produced marked stimulation of growth in concentrations even up to 4 per cent. In concentrations of 6 and 8 per cent the colonies still appear larger than the control. If, therefore, colony area alone were taken as a means of measuring growth, it could be concluded that even in these percentages stimulation of growth has occurred. When thinner densities as well as area measurements are taken into consideration, it must be concluded that stimulation is effected only by concentrations of 4 per cent or less of Fraction $7B_2$. From a comparison of $7A_2$ and $7B_1$, it might appear that the growth factor is not appreciably adsorbed by charcoal. However, when $7B_1$ is freed of oil content, it shows better growth-promotion than either $7A_1$ (crude) or $7A_2$ (unadsorbed).

Other fractions, prepared in essentially the same way as far as acetone extraction is concerned but differing somewhat in the procedure of purification, were also tested on the growth of E. coli. In the case of Fractions 8C and 8B, the charcoal adsorption step was omitted and an extraction with ether and chloroform was made to eliminate the fatty substances from an acetone fraction which corresponded to Fraction $7A_1$. The fatty fraction was called 8C, the fat-free fraction, 8B. The cultural characters of the colonies grown on the media containing the various concentrations of these fractions are presented in the first two columns of Plate II. All the experimental colonies grown on the media containing Fraction 8C differ markedly from the control both in the configuration and the nature of the growth. A change in the length of the organism from short rods, 1 to 3 microns long, to rods 6 to 10 microns long, was observed even in smears made of organisms grown on media containing only 1 per cent of Fraction 8C. The fact that stimulation of growth occurred even in the higher concentrations of Fraction 8C leads us to think that the chloroform-ether extraction, while removing the fatty substance, did not effect a complete separation of the growth-promoting and growth-depressing factors. That there was indeed some separation is evidenced by the luxuriant growth noted in the cultures made of E. coli on nutrient agar containing varying concentrations of the fat-free Fraction 8B. Stimulation of growth was effected in all the concentrations used. However, concentrations from 4 to 8 per cent changed the colonies from forms characteristic of E. coli to the R-type of colony with roughened surface and granular consistency. In the microscopic examination of the organisms grown on the media containing Fraction 8B, transformation to filamentous forms, together with the persistence of individual organisms, was noted. The individual forms, while decreasing in number, were never entirely absent even on the media

containing the highest concentrations. The growth in this respect differed from that on the media containing 4 to 10 per cent of the acetone fractions previously discussed.

Since the tests made of the growth-promoting power of Fraction $7B_2$ showed that a considerable amount of the growth factor had been adsorbed on the charcoal, it was decided to determine if this growth factor could still be obtained after the alcoholic extract had been adsorbed on Norit charcoal. Accordingly, an extract corresponding to 7C (an alcoholic extract which will be discussed later) was adsorbed on Norit charcoal and the adsorbed portion eluted with 50 per cent acetone. This preparation is summarized in Fig. 1 and is designated as Fraction S35. The first two experimental colonies (0.5 and 1 per cent) showed a marked stimulation in the normal growth of E. coli. In the next two higher concentrations (2 and 4 per cent) stimulation of growth still prevailed. However, the nature of the growth could no longer be considered normal. There was a considerable change in the character of the colonies, from that characteristic of E. coli to the R-type. In the media containing the two highest concentrations there was a marked falling off in the growth of the colonies after the first day of incubation. The succeeding growth appeared rather transparent and beady. A few filamentous forms were observed on microscopic slides made of the organisms from these two cultures, though the short bacillus form was also present in great numbers.

Table I presents the area measurements of colonies grown on nutrient agar modified by varying concentrations of the three fat-free fractions, $7B_2$, 8B, and S35, which were derived by the different methods already referred to. In this case area measurements may be considered proportional to the growth since the colonies showed comparatively equal densities. All three fractions exhibit maximum effect on colony size in concentrations of 2 per cent. If we compare the stimulating

TABLE I

Effect of Fat-Free Fractions of Acetone Yeast Extracts on the Growth of Escherichia Coli

	Area of Colony in Square Centimeters							
	Control	0.5%	1%	2%	4%	6%	8%	10%
$7B_2$	0.76	1.10	1.55	2.61	1.44	0.94	1.22	0.26
8B	0.39	1.10	1.16	2.77	2.51	1.41	1.16	
S35	0.45	1.06	1.90	4.35	2.00	0.87	1.00	
	Per Cent Increase in Area Over Control							
$7B_2$		44	104	244	89.3	23.4	6.1	−65
8B		183	200	600	550	266	200	
S35		135	321	864	343	92	121	

powers of these fractions as indicated by the per cent increase in areas of the colonies, it is noted that 8B is 2.5 times, and S35 is 3.5 times as effective as $7B_2$.

Three alcoholic fractions were also tested on the growth of E. coli. Fractions 7C (Plate I) and 2A (Plate II) were prepared in the same way from two different batches of yeast. It is clear from Plate I that the colonies of Fraction 7C, while becoming larger on media containing 0.5 to 2 per cent of this fraction, differ little in configuration and texture from the control colonies. The inhibition of growth in the higher concentrations is also less. It is significant in this regard to point out that the variation from short rods to filaments 15 to 20 microns long occurred only in the cultures on media in which 8 and 10 per cent of the fraction had been incorporated. These short branched filaments were suggestive of the branching

Y forms described by Gardner[7]. With these, the normal short rods were also present in greater numbers.

This same observation concerning morphology was true of E. coli grown on media containing the alcoholic extract, Fraction 2A. The very irregularly shaped colonies (Plate II, 2A) were due to an excess of moisture on the surface of the agar plates when the colonies were planted. The irregularity of the colonies in these cases had no connection with the altered morphology of E. coli. As mentioned above, the microscopic examination of the stained smears indicated no pleomorphism except in the organisms grown on the media containing 8 per cent of Fraction 2A.

The third alcoholic extract, Bio 23, was derived by an immediate extraction of the yeast with ethyl alcohol denatured with 10 per cent methanol. No previous extraction with acetone had been made. The growth-promoting property of this fraction peaked at a concentration of 6 per cent and then suddenly dropped. Simultaneously with this drop in growth the occurrence of the long filaments was noticed.

The results obtained from the study of E. coli which had been cultivated in nutrient broth containing the alcoholic fractions, 2A and Bio 23, indicate a stimulation of growth in all the concentrations (0.5 to 10 per cent). The growth was maximum in concentrations of 2 per cent. The acetone fractions effected stimulation of growth in concentrations up to 6 per cent with a maximum at 1 and 2 per cent.

Transformation to filamentous forms which occurred on solid media did not occur in any of the liquid cultures.

The pleomorphic forms produced in all the experiments were not variants but rather responses to current conditions. This was proved by the reversion of the altered forms to normal E. coli when they were transplanted to ordinary nutrient agar and nutrient broth. Warm stage studies of pieces of filamentous growth in a hanging drop of nutrient broth suggested the

development of spore-like gonidia within the filaments. Evidence for this is the following: After the hanging drop in which the filaments were suspended had been incubated at 37.5° C. for about 10 hours, the filaments began to display movement apparently caused by some force within them. A little later short, plump, highly motile bacilli were noticed moving rapidly across the field. After about 6 more hours the movement of the filaments ceased. By this time the microscopic field was swarming with great numbers of highly motile rods. When these were subsequently transplanted to nutrient agar they were identified both by staining reaction and biochemical tests as the original E. coli.

It is not possible at present to predict the extent to which the fractions investigated may be used as a means of stimulating the growth of other microorganisms.

SUMMARY AND CONCLUSIONS

Escherichia coli was used to measure the effect on bacterial growth of varying concentrations of acetone and alcoholic fractions of yeast extracts.

The macroscopic and microscopic study made of the cultures indicates that the acetone-soluble fractions in concentrations from 0.5 to 2 per cent stimulate growth, and that an appreciable quantity of the growth-promoting material is adsorbed by charcoal. Higher concentrations (4 to 10 per cent) in the solid media not only altered the normal form of the colonies, but likewise affected the morphology of E. coli, causing it to produce increasingly longer rods and finally a network of long branching filaments 50 and more times the length of the ordinary bacillus. Transplanted to plain nutrient agar and nutrient broth, the filaments again produced normal E. coli. The changes are accounted for by the probable formation of spore-like gonidia within the filamentous branching forms when the concentration of the fractions became so great as to

constitute an unfavorable environment for the organism. The alcohol-soluble fractions stimulated growth in concentrations from 0.5 to 6 per cent. Filaments similar to those observed in the cultures on media containing the acetone fractions were produced only in cultures on media in which had been incorporated 8 and 10 per cent of the alcoholic fractions. The flourishing bacterial growth as represented by E. coli suggests the use of the more active fractions in suitable concentration for enhancing the growth of other bacteria which are more fastidious in their nutritional requirements. It cannot be stated at this time whether the depression of growth by the higher concentrations is simply a concentration effect or is due to the presence of inhibitory substances.

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LITERATURE CITED

Cook, E. S., Kreke, C. W., Giersch, Sr. M. C., and Schroeder, Sr. M. P., *Science*, **93** (1941): 616.

Gardner, A. D., *J. Path. Bact.*, **28** (1925): 189.

Hata, S., *Centr. Bakt. Parasitenk.*, **46** (1908): 289.

Henrici, A. T., *J. Infectious Diseases*, **38** (1926): 54; **39** (1926): 429.

Reed, G., and Orr, J. H., *J. Bact.*, **8** (1923): 103.

Vay, F., *Centr. Bakt. Parasitenk.*, **55** (1910): 193.

Wilson, W. J., *J. Path. Bact.*, **11** (1906): 394.

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COMPARATIVE GROWTH STUDIES ON A PROLIFERATION-PROMOTING EXTRACT FROM ULTRAVIOLET INJURED YEAST CELLS, KNOWN BIOS COMPONENTS AND AMINO ACIDS*

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Previous papers from these laboratories have shown that the subjection of yeast, other micro-organisms, and a variety of animal tissues to diverse forms of injury, such as ultraviolet irradiation, x-rays, mechanical injury, chemical irritation and oxygen lack, results in the release into the intercellular fluids of substances which stimulate cellular proliferation.[1, 2, 3] These proliferation-promoting substances would appear to be "wound hormones" in the original sense proposed by the botanist Wiesner[4] and dealt with later by a number of writers. These materials appear to be produced or released by living cells into the intercellular fluid as a result of injury and hence have been

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[1]Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sr. M. V., *Nature*, **139** (1937): 589; Fardon, J. C., and Sullivan, W. A., These Studies, **2** (1938): 39; Fardon, J. C., Sullivan, W. A., and Andrus, Sr. M. B., *ibid.*, **2** (1939): 233; Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M., *Nature*, **140** (1937): 643; Loofbourow, J. R., and Dwyer, Sr. C. M., *Science*, **88** (1938): 191; *Nature*, **143** (1939): 725; These Studies, **2** (1939): 155; Loofbourow, J. R., Cueto, A. A., and Lane, Sr. M. M., *Arch. exptl. Zellforsch.*, **22** (1939): 607; Loofbourow, J. R., Cook, E. S., Dwyer, Sr. C. M., and Hart, Sr. M. J., *Nature*, **144** (1939): 553; Loofbourow, J. R., and Morgan, Sr. M. N., *J. Bact.*, **39** (1940): 437.

[2]Loofbourow, J. R., Dwyer, Sr. C. M., and Morgan, Sr. M. N., These Studies, **2** (1938): 137.

[3]Loofbourow, J. R., Dwyer, Sr. C. M., and Lane, Sr. M. M., *Biochem. J.*, **34** (1940): 432.

[4]Wiesner, J., Die Elementarstructur und das Wachstum der lebenden Substanz, Vienna, 1892.

called "intercellular hormones."[5] The reasons for this belief have been summarized recently.[6] The subject of wound hormones is treated extensively in reviews now in preparation[7] and need not be discussed further in this place.

Certain questions naturally arise as to the nature of the proliferants produced by injury, e.g., whether they merely represent cellular destruction products which serve as simple nutrients; whether they represent an increased production of normal growth factors, such as the bios group for yeast; or whether they consist of a different type of substance which may be considered to be formed or released more or less as a specific response to injury. While crude preparations from injured cells contain a certain amount of cellular destruction products, by controlled injury with radiations it is possible to hold this material to a minimum, and the papers cited in the preceding paragraph furnish evidence that such cellular debris does not play the main role in the growth activity (see particularly[6, 7] for summaries).

Early in the study of crude cell-free filtrates from injured yeast and animal tissue cells it was found that these preparations were characterized by marked ultraviolet absorption at approximately 2600 Å and that the extinction coefficient paralleled the proliferation potency of these materials[3, 8]. This absorption is characteristic of the purine and pyrimidine components of nucleic acids and it was believed that the increased growth activity might be due to the increased formation of such substances, this increased formation being

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[5]Loofbourow, J. R., and Morgan, Sr. M. N., THESE STUDIES, 2 (1938): 113.

[6]Loofbourow, J. R., Dwyer, Sr. C. M., and Cronin, Sr. A. G., *Biochem. J.*, 35 (1941): 603.

[7]Cook, E. S., and Fardon, J. C., in preparation; Loofbourow, J. R., *Biol. Rev.*, in preparation.

[8]Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, 142 (1938): 573; Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° congr. intern. chim.*, 5 (1939): 26.

confirmed by chemical findings.[8] This appeared to be a reasonable assumption since Caspersson[9] has noted an increase of nuclear materials during cell division and Fischer[10] considers a nucleoprotein to be the growth substance involved in his studies of tissue proliferation. It has further been possible, by means of the quartz microscopic technique, to demonstrate an increase in materials absorbing at 2600 Å during the ultraviolet irradiation of living yeast cells,[11] and other work[12] indicates an increase in the amount of material isolable as nucleic acid by the procedure of Johnson and Harkins[13] from yeast irradiated with ultraviolet. Finally, Davidson[14] has confirmed the proliferative properties of cell-free filtrates from ultraviolet treated yeast cell suspensions and by quantitative analytical procedures he has demonstrated that these filtrates contain greatly increased nucleotide-, nucleoside-, and free purine-nitrogen, but very little protein, thus substantiating our spectroscopic and chemical evidence. From these findings there can be no doubt that an increase in nucleic acid-like materials results when cells are subjected to injuring processes such as ultraviolet irradiation. Should these substances be responsible for the proliferative effects observed on yeast, as seemed likely from the spectroscopic evidence, they would have to be regarded as distinct from the currently recognized bios components which, with the possible exception of uracil,[15] do not have the proper ultraviolet absorption characteristics.[16]

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[9]Caspersson, T., *Skand. Arch. Physiol.*, **73**, Supplement No. 8, (1936); Caspersson, T., and Schultz, J., *Nature*, **142** (1938): 294; **143** (1939): 602.

[10]Fischer, A., *Nature*, **144** (1939): 113; *Chemical Products*, **3** (1940): 79.

[11]Loofbourow, J. R., and Joyce, L., *Nature*, **148** (1941): 166.

[12]Loofbourow, J. R., Englert, Sr. M. E., and Dwyer, Sr. C. M., *Nature*, **148** (1941): 113.

[13]Johnson, T. B., and Harkins, H. H., *J. Am. Chem. Soc.*, **51** (1929): 1779.

[14]Davidson, J. N., *Biochem. J.*, **34** (1940): 1537.

[15]Williams, R. J., *Biol. Rev.*, **16** (1940): 49.

[16]Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, **34** (1940): 1580.

This possibility was emphasized by the fact that "bios" fractions prepared by classical procedures show no correlation between their growth potency and their ultraviolet absorption at 2600 Å.[16]

It is essential, however, that a comparison be made of the proliferative preparations from injured cells with the known bios factors for yeast since the crude cell-free extracts might be expected to owe a considerable part of their activity to a content of some of these previously identified materials known to be abundantly present in yeast. This comparison is further required by certain other facts currently under investigation. Crude nucleic acid from ultraviolet treated yeast cells possesses growth promoting properties[12] which appear to be lacking in pure nucleic acid.[3] The purification of preparations from injured cells by the technique of chromatographic adsorption has furnished very potent proliferation-promoting concentrates which lack the characteristic ultraviolet absorption. While these results will be published later, they suggest that the growth activity of the crude extracts from injured cells may be due to substances other than the ultraviolet absorbing ones although the latter would appear to have been produced concomitantly. Quite evidently a comparison with the known proliferants is in order.

The definitely recognized substances which act as bios components for various types of yeast are inositol, thiamin, biotin, pantothenic acid and vitamin B_6 (see excellent review of Williams.[15]) Certain amino acids have for a long time also been implicated in the bios problem, and their importance for yeast growth has been emphasized by recent papers of Nielsen and Hartelius[17] and of Mitchell and Williams.[18] The position of the amino acids assumes possible additional significance for

[17]Nielsen, N., and Hartelius, V., *Biochem. Z.*, **295** (1938): 211, and later papers.

[18]Mitchell, H. K., and Williams, R. J., *Biochem. J.*, **34** (1940): 1532.

our problem since Davidson, in the paper referred to,[14] found the filtrates from ultraviolet injured yeast to be relatively rich in amino-nitrogen. Previously a number of these substances together with certain others (nicotinic acid, adenosine phosphate, riboflavin) were tested individually and found to be without significant effect on the growth of yeast under our experimental conditions.[3] However, many of these materials exert their maximum effect only in combination, and it is further essential that the wound hormone preparations be assayed in media adequate in known factors rather than simply in Reader's medium[19] as in our earlier work. Accordingly, a cell-free filtrate from an ultraviolet irradiated yeast suspension was assayed for its effect on the growth of yeast in the following media: 1) Reader's medium; 2) Reader's medium plus a bios supplement (inositol, thiamin, biotin, pantothenic acid and vitamin B_6); 3) Reader's medium plus the bios supplement plus an amino acid supplement (containing twenty amino acids); 4) Williams' medium[20]; 5) Williams' medium plus the bios supplement; 6) Williams' medium plus the bios supplement plus the amino acid supplement (asparagin being omitted from the Williams' medium in this case).

EXPERIMENTAL

Wound Hormone Preparation

This was prepared in a manner similar to that described earlier[2] by suspending 917 grams of Fleischmann's bakers' yeast in ten liters of 0.85 per cent. sodium chloride solution and irradiating this suspension at 20 cm. with full ultraviolet from a Burdick quartz mercury arc operated at 55 volts and with constant stirring. The irradiation was continued for 27 hours, at the end of which time approximately 60 per cent.

[19]Reader, V., *ibid.*, **21** (1927): 901.

[20]Williams, R. J., and Saunders, D. H., *ibid.*, **28** (1934): 1887.

of the cells were killed, as indicated by methylene blue staining. The suspending fluid was separated from the cells by centrifuging, followed by Büchner filtration using Filter-Cel, and finally by Berkefeld filtration. The solution was concentrated to 610 cc. *in vacuo* and autoclaved at 20 pounds for 20 minutes. A brown precipitate was filtered off and discarded and the filtrate was passed through a Berkefeld filter. The solid content was obtained by evaporation of a measured sample to dryness and was corrected for salt which was determined by titration with silver nitrate, using dichlorofluorescein as an indicator. The final concentrate contained 28 mg. of solids (non-salt) per cc. This material was added in serial dilutions to the growth tubes.

Media

Reader's medium[19] and Williams' medium[20] were prepared as directed by the authors.

Bios Supplement

The following materials were used: inositol (Eastman), thiamin hydrochloride (Merck), biotin (as a concentrate from S. M. A. Corporation containing 100 γ of biotin per cc.), calcium (+) pantothenate (gift of Merck and Co.), and vitamin B_6 hydrochloride (gift of Merck and Co.). These were dissolved in sterile distilled water in the ratio indicated by Williams *et al.*[21] to form a stock solution which could be added in convenient amounts to the media in the assay tubes. Preliminary experiments, which are not reported in detail, were carried out to determine for our yeast and under our conditions the adequate concentrations of the pure bios components. These concentrations, in γ per cc., were found to be: inositol, 100; thiamin hydrochloride, 0.8; biotin, 0.002; calcium (+) pantothenate, 0.12; vitamin B_6 hydrochloride, 0.8. These concentrations, which held for both Reader's and Williams'

media, are, if anything, somewhat in excess of the necessary amounts, quantities only 0.6 of the above giving, within experimental error, practically as good growth. For example, in Reader's medium the lower quantity of the bios supplement gave 0.65 mg. of yeast as compared with 0.70 mg. obtained with the higher quantity.

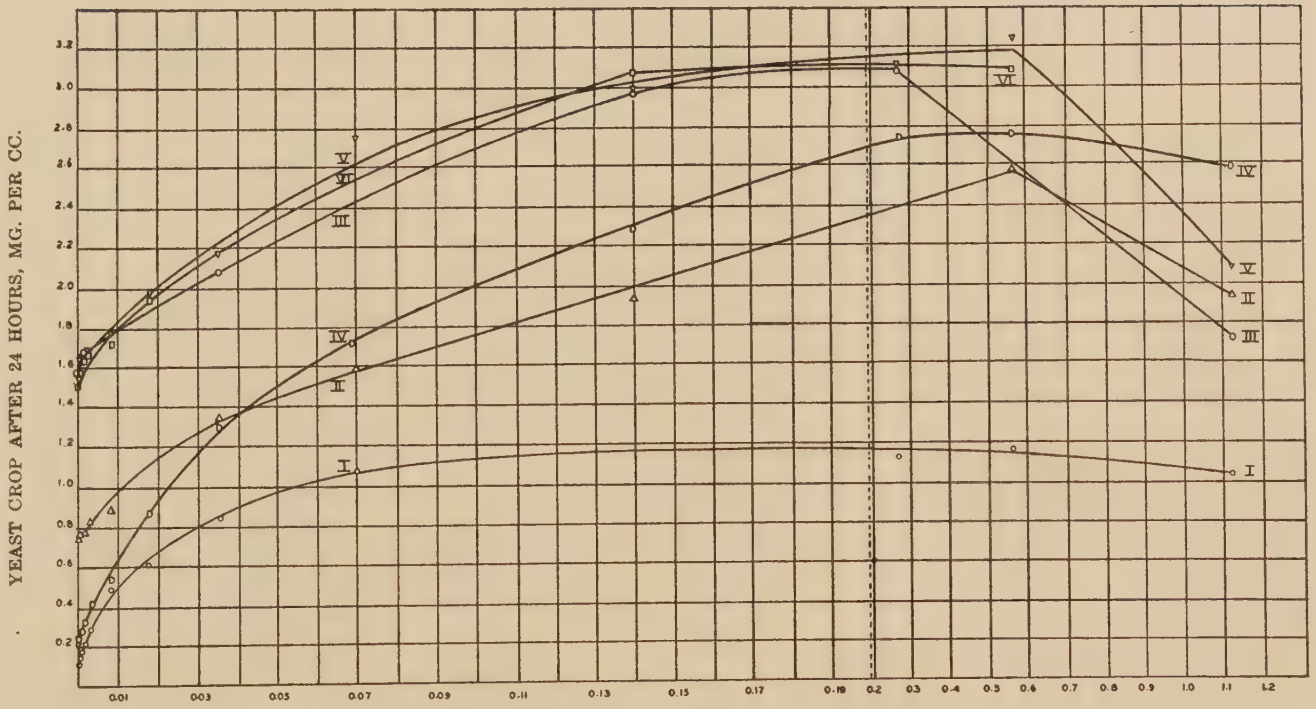
Amino Acid Supplement

Based on the work of Mitchell and Williams[18] sterile stock solutions were prepared containing equal weights of the following twenty amino acids (Eastman): asparagine, alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine. Preliminary experiments were carried out to ascertain optimum quantities of the acids. In these experiments the concentration of each acid in the mixture was varied from 0.06 mg. per cc. to 0.48 mg. per cc. (total acids from 1.2 mg. to 9.6 mg. per cc.). No advantageous effect on growth was obtained by adding the highest as compared with the lowest amino acid concentration. (Lowest concentration crops: Reader's, 1.54 mg.; Williams', 1.51 mg. Highest concentration crops: Reader's, 1.57 mg.; Williams', 1.56 mg.).

Growth Assays

These were carried out according to the rocker-tube technique previously employed in our laboratories,[2, 3, 16] with the single exception that yeast crops are expressed in terms of dry weight of yeast instead of wet weight. *Saccharomyces cerevisiae*, grown from a single cell isolated from Fleischmann's bakers' yeast, was washed from 72-hour slants on Sabouraud's medium and made up in isotonic salt solution in such a concentration that 1 cc. diluted to 25 cc. in the rocker-tube gave a yeast concentration of 0.03 mg./cc. (dry weight; corresponds approximately

FIG. 1—*Effect of Wound Hormone Preparation on Yeast Growth in Various Media*



I. Reader's medium. II. Reader's medium plus bios supplement. III. Reader's medium plus bios supplement plus amino acid supplement. IV. Williams' medium. V. Williams' medium plus bios supplement. VI. Williams' medium plus bios supplement plus amino acid supplement.

to 0.064 mg. wet weight used previously). The concentration of yeast used for seeding and the crop after 24 hours at 30° C. were determined by means of a photoelectric densitometer.

RESULTS AND DISCUSSION

The results of typical experiments are shown in Fig. 1. The curves in this figure represent the effects of different amounts of the wound hormone preparation on the 24 hour crop of *S. cerevisiae* under the previously specified growth conditions and in different media. The following media are represented by the curves:

I. Reader's medium.

II. Reader's medium plus bios supplement (γ per cc.: inositol, 100; thiamin chloride, 0.8; biotin, 0.002; calcium (+) pantothenate, 0.12; vitamin B_6, 0.8).

III. Reader's medium plus bios supplement plus twenty amino acids (total of 1.2 mg. per cc.).

IV. William's medium.

V. William's medium plus bios supplement.

VI. Williams' medium plus bios supplement plus amino acids.

Inspection of the curves reveals that, in the absence of the wound hormone, Reader's medium alone allows the least growth; addition of the bios supplement increases the growth approximately 5.5 times; and addition of both bios and amino acid supplements increases the growth nearly 11 times. In the absence of the wound hormone Williams' medium is superior to Reader's, giving nearly 1.8 times as good growth. Addition of the bios supplement improves the growth in Williams' medium about 6.8 times. Addition of the amino acids resulted in no further improvement. This last fact, as well as a large part of the superiority of Williams' over Reader's medium,

probably is due to the presence of asparagin in the former. An experiment using Williams' medium without asparagin showed it to have but very slight superiority over Reader's when the bios supplement was added to both (crop of 0.72 mg. of yeast as compared with 0.65 mg.).

In all the media addition of the crude wound hormone preparation resulted in increased growth, definitely detectable effects being exerted by 0.5 γ per cc. of this preparation, the lowest concentration employed.

In order to compare the effects of the wound hormone preparation in different media it is convenient to express the results in terms of growth units. In the earlier publications from these laboratories such a unit was defined in terms of an absolute increment in yeast weight or count. Specifically, a growth unit represents an increase in yeast crop of 0.747 mg. (dry weight) per cc. greater than that in the control. As we have pointed out,[16] such a method of comparing growth potencies is strictly valid only when the growth-concentration curves are linear. None the less, with similar portions of approximately similar curves this unit has proven to be a reasonably satisfactory means of comparison for materials assayed in the same basic medium. In the present experiments, however, such a unit shows a defect. So long as the same basic growth medium is being used, the absolute-increment unit will always represent approximately the same percentage increase in yeast crop. However, when different basic media are being used for the comparative assay of an unknown material, as in the present work, an absolute-increment unit will represent varying percentages of the control crop and may therefore result in misleading interpretations of the growth data. Hence, we have also calculated the growth data in terms of a relative unit which merely represents an increase in experimental yeast crop of 100 per cent. over that of the control. This unit, while varying in absolute value for the different media, is useful for

TABLE I

Potencies of Wound Hormone Preparation in Different Media

Medium*	Growth Units (Absolute) per mg.**	Growth Units (Relative) per mg.†
I	27	286
II	20	22
III	18	7
IV	44	200
V	25	7
VI	23	8

*See text for composition.

**1 g.u. (absolute) represents increase in yeast crop of 0.747 mg./cc. greater than that in control.

†1 g.u. (relative) represents a 100 per cent. increase in yeast crop as compared with control.

comparison of the effects of the wound hormone preparation in the different media. The use of this unit puts the wound hormone preparations in the worst light since the better media require a much greater crop of yeast per unit of activity. It is obvious that neither of these units is wholly satisfactory and attention must be directed to the growth curves as a whole in studying the relative potencies.

In Table I are given the proliferation-promoting potency of the crude wound hormone preparation in absolute and relative units per mg. in the different media.

If the absolute units are considered, the wound hormone preparation exhibits an apparently greater activity in plain Williams' medium than in Reader's. This is somewhat contrary to expectations, since Williams' medium is more complete, unless a supplementary action is being exhibited. The expected lower activity is found when the relative units are used as a basis of comparison.

Addition of the known bios factors decreases the relative effectiveness of the wound hormone preparation, but this decrease is comparatively slight in terms of the absolute units. On the other hand, use of the relative units indicates that about 92 per cent. of the activity of the wound hormone preparation in Reader's medium is due to the presence of the bios factors. The additional lowering of potency in Williams' medium is due largely to the presence of asparagin since the wound hormone preparation assayed in Williams' medium without asparagin but with the bios supplement, showed an activity of 17 growth units (relative) per mg.

Addition of the amino acids only very slightly lessens the effectiveness of the wound hormone preparation in either Reader's or Williams' media according to the absolute units. In terms of the relative units, the amino acids decrease the "potency" of the yeast preparation by approximately a further 5 per cent. in Reader's medium but have no effect in Williams', the latter fact again, no doubt, being due to the asparagin. The importance of amino acids for optimum yeast growth, as found by Hartelius[17] and Williams,[18] is thus confirmed, and quantitatively the figures support Williams' finding[18] that Fleischmann's bakers' yeast is considerably less demanding in its amino acid requirements than are some other strains. It will be recalled that Davidson[14] found irradiated yeast extracts to contain an appreciable amount of amino nitrogen. Assuming an average molecular weight of our 20 amino acids to be 142 and assuming only one amino group to be present, our media contained from 12 to 96 mg. of amino nitrogen per cc. Davidson found 63 mg. per cc. in his irradiated yeast preparations, a figure lying between the extreme quantities used by us. It is thus apparent that a certain, although relatively slight, amount of the activity of the irradiated yeast filtrates is due to the amino acid content, and, inferentially, asparagin is an acid of leading importance.

We must conclude that a definite portion of proliferation-promoting activity for yeast (*S. cerevisiae*, Fleischmann bakers') of extracts from ultraviolet irradiated yeast is dependent upon their content of the known bios factors and to a lesser extent upon their amino acid content. There is, however, a definite and significant residual activity which must be due to factors as yet unidentified. This activity is readily detectable in concentrations of crude extract as low as 0.5 γ per cc. This is in accord with our earlier suggestion[16] and the belief of Williams[21] that tissue extracts contain growth-promoting substances for yeast other than the known bioses. We should also call attention to the fact that the wound hormone preparation, in its highest concentration, shows evidence of toxicity, this effect being least pronounced in the unsupplemented media and thus indicating that too high a concentration of certain constituents is unfavorable.

Preliminary experiments were also carried out on the effects of calcium (+) pantothenate (in concentrations of from 0.05 to 1 mg. per cc.); of biotin (in concentrations of from 0.0135 to 0.027 γ per cc.); of the bios supplement and of the amino acid supplement, alone and combined; and of the irradiated yeast extract on the respiration of the same strain of *S. cerevisiae* in M/15 KH_2PO_4 containing 0.02 per cent. glucose, using the Warburg method. While the yeast extract caused a small increase in oxygen uptake (of the order of 25 per cent.) in a one-hour period in concentrations of 0.5-1.0 mg. per cc., the pure compounds gave no increase in respiration. Previously[22] we had found a number of amino acids, studied individually, nicotinic acid, riboflavin, vitamin B_6, inositol and several other substances to be without effect, or to have only a very slight

[21] Williams, R. J., Eakin, R. E., and Snell, E. E., *J. Am. Chem. Soc.*, **62** (1940): 1204.

[22] Cook, E. S., Walter, E. M., and Eilert, Sr. M. R., *Proc. Soc. Exptl. Biol. Med.*, **44** (1940): 547.

effect, on the respiration of bakers' yeast. Only thiamin had a somewhat greater action in relatively high concentrations. These experiments suggest a measure of independence between the respiration and growth processes, or, perhaps more correctly, an indirect interdependence of the two processes. It seems probable that an actual deficiency in certain of these substances would be manifested in a lowered respiration of the yeast since certain of the known growth substances are constituents of cellular respiration systems. The respiration of pantothenic acid-deficient yeast may be stimulated by the addition of pantothenic acid[23] and we have encountered suggestions of a similar behavior with regard to other substances in earlier work.[22] The relation of growth substances to cellular respiration is receiving further investigation.

The work reported in this paper, of course, does not offer suggestions as to the nature of the new proliferants involved. A few compounds not considered in this paper, such as uracil, ethanolamine, nicotinic acid, riboflavin and choline, have recently been reported to have some influence on the growth of some strains of yeast.[15] While these must be considered in future work, available evidence renders it improbable that they will play a large part in the growth activity of the irradiated yeast extracts. Nicotinic acid and riboflavin have no effect on our yeast in Reader's medium.[3] Studies of this problem, with attempts at concentration and identification of the active materials, are in progress. Our earlier beliefs with regard to the possible nucleic acid-like nature of the proliferant are summarized in the introduction to this paper.

Another problem closely related to the above is whether injury causes cells to produce or release specific proliferants or whether the wound hormones merely represent an increased production of normal growth promoters. As pointed out earlier

[23]Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, **22** (1939): 637.

in this paper, the correlation of the ultraviolet absorption at 2600 Å with the biological potency of the wound hormone preparations led us to suspect that their activity was due for the most part to materials having those absorption characteristics and, hence, not of the recognized bios type. While the release of such substances as a result of injury cannot be doubted, it is by no means certain that the proliferation potency can be attributed solely to these substances. The present results suggest that injury may also cause the release (and perhaps increased synthesis) of the normal growth substances. It is evident that extracts from injured and "uninjured" yeast and tissues both contain substances in addition to the known bioses which stimulate the proliferation of yeast.[21] For example, an extract from unirradiated yeast cells prepared as a control to the extract from irradiated cells used in this paper had a potency of 12 absolute or 145 relative growth units per mg. when assayed in Reader's medium. In our present state of information it seems best to assume that the "wound hormones" with which we have been dealing represent in part normal growth substances although it is likely that specific substances released as an injury response may be involved.

The present status of the wound hormone problem with respect to yeast may be summarized as follows:

1. Yeast and tissue extracts contain growth factors or nutrilites for yeast which differ from the recognized bios components.

2. Injury to cells by diverse means causes an increased production or release of growth factors for yeast, a portion of the activity apparently being due to the known bios components and amino acids, with, however, a definite portion being due to unknown materials.

3. Injury to cells causes an increased release, and probably production, of nucleic acid-like materials absorbing ultraviolet at approximately 2600 Å.

4. The possible relationship of the nucleic acid-like materials to the normal nutrilites or to nutrilites which may be more or less specifically formed as a response to injury is not completely established although there appears to be a relationship between the production of these materials and the injury process.

SUMMARY

The ability of cell-free filtrates from ultraviolet injured yeast cells (crude "wound hormone" preparations) to stimulate the proliferation of *Saccharomyces cerevisiae* (Fleischmann's bakers' yeast) has been studied in Reader's and Williams' media with and without the addition of known bios components (inositol, thiamin, biotin, pantothenic acid and vitamin B_6) and of twenty amino acids.

A significant portion of the growth activity is due to these known factors but a definite portion of the potency is due to a material or materials other than these.

The possible relationship of nucleic acid-like materials, selectively absorbing ultraviolet at about 2600 Å, to the wound hormones is discussed.

The amino acids and the bios components, with the exception of thiamin, for the most part exert no or only a slight effect on the respiration of our yeast, and the wound hormone preparation causes only a 25 per cent. stimulation in concentrations of 0.5 to 1.0 mg. per cc.

NOTE ADDED IN PROOF

Since the preparation of this manuscript we have observed that the twenty amino acids may be replaced with almost complete satisfaction by the six acids: arginine, asparagine, asparatic acid, glutamic acid, leucine and lysine. Also we have further supplemented the media with the following (in γ per cc.): riboflavin, 1; uracil, 1; choline hydrochloride, 1.5; acetyl choline bromide, 1; ethanolamine, 5; nicotinic acid, 5. The

addition of these substances seems to have slight or no effect on the growth of our strain of yeast and they do not lessen the activity of the wound hormone preparation. Addition of ρ-aminobenzoic acid in concentrations of 2.5 to 10 γ per cc. did not increase the yeast crop and, in some concentrations, gave a suggestion of slight inhibition, which may not be significant.

BIBLIOGRAPHY

Caspersson, T., *Skand. Arch. Physiol.*, **73**, Supplement No. 8, (1936).

Caspersson, T., and Schultz, J., *Nature*, **142** (1938): 294; **143** (1939): 602.

Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M., *Biochem. J.*, **34** (1940): 1580.

Cook, E. S., Loofbourow, J. R., and Stimson, Sr. M. M., *Atti X° congr. intern. chim.*, **5** (1939): 26.

Cook, E. S., Walter, E. M., and Eilert, Sr. M. R., *Proc. Soc. Exptl. Biol. Med.*, **44** (1940): 547.

Davidson, J. N., *Biochem. J.*, **34** (1940): 1537.

Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, M. V., *Nature*, **139** (1937): 589.

Fardon, J. C., and Sullivan, W. A., THESE STUDIES, **2** (1938): 39.

Fardon, J. C., Sullivan, W. A., and Andrus, Sr. M. B., THESE STUDIES, **2** (1939): 233.

Fischer, A., *Nature*, **144** (1939): 113; *Chemical Products*, **3** (1940): 79.

Johnson, T. B., and Harkins, H. H., *J. Am. Chem. Soc.*, **51** (1929): 1779.

Loofbourow, J. R., Cook, E. S., Dwyer, Sr. C. M., and Hart, Sr. M. J., *Nature*, **144** (1939): 553.

Loofbourow, J. R., Cook, E. S., and Stimson, Sr. M. M., *Nature*, **142** (1938): 573.

Loofbourow, J. R., Cueto, A. A., and Lane, Sr. M. M., *Arch. exptl. Zellforsch.*, **22** (1939): 607.

Loofbourow, J. R., and Dwyer, Sr. C. M., *Science*, **88** (1938): 191; *Nature*, **143** (1939): 725; THESE STUDIES, **2** (1939): 155.

Loofbourow, J. R., Dwyer, Sr. C. M., and Cronin, Sr. A. G., *Biochem. J.*, **35** (1941): 603.

Loofbourow, J. R., Dwyer, Sr. C. M., and Lane, Sr. M. M., *Biochem. J.*, **34** (1940): 432.

Loofbourow, J. R., Dwyer, Sr. C. M., and Morgan, Sr. M. H., THESE STUDIES, 2 (1938): 137.

Loofbourow, J. R., Englert, Sr. M. E., and Dwyer, Sr. C. M., *Nature*, 148 (1941): 113.

Loofbourow, J. R., and Joyce, L., *Nature*, 148 (1941): 166.

Loofbourow, J. R., and Morgan, Sr. M. N., *J. Bact.*, 39 (1940): 437; THESE STUDIES, 2 (1938): 113.

Mitchell, H. K., and Williams, R. J., *Biochem. J.*, 34 (1940): 1532.

Nielsen, N., and Hartelius, V., *Biochem. Z.*, 295 (1938): 211, and later papers.

Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, 22 (1939): 637.

Reader, V., *Biochem. J.*, 21 (1927): 901.

Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M., *Nature*, 140 (1937): 643.

Wiesner, J., Die Elementarstructur und das Wachstum der lebenden Substanz, Vienna, 1892.

Williams, R. J., *Biol. Rev.*, 16 (1940): 49.

Williams, R. J., Eakin, R. E., and Snell, E. E., *J. Am. Chem. Soc.*, 62 (1940): 1204.

Williams, R. J., and Saunders, D. H., *Biochem. J.*, 28 (1934): 1887.

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